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BIOLOGICAL AND CHEMICAL STUDIES OF THE LACTOBACILLUS GENUS WITH SPECIAL REFERENCE TO XYLOSE FERMENTATION BY L. PENTOACETICUS[1]

LOUIS WEINSTEIN AND LEO F RETTGER

Laboratory of Bacteriology, Yale University

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Up to within the present decade, no work had been done with microorganisms which produce comparatively large amounts of lactic and acetic acids from the pentose, xylose. Scattered references are found in the literature preceding 1919 to the ability of various organisms to attack xylose.

Stone (1890) and others claimed that yeasts alone are unable to ferment xylose. Later workers confirmed these observations, but found that yeasts assimilate a small amount of xylose in a culture medium containing this pentose. On the other hand, the production of lactic acid from xylose by the bacteria in pressed yeast was demonstrated. According to Went (1901) molds do not ferment xylose. Malenkowitsch (1906) studied the action in rotting wood of the molds *Merilius lacrymans* and *Conophora cerebella* and was unable to show that they attack xylan.

Grimbert (1896) discovered that the pneumobacillus of Friedlander converts xylose into alcohol, acetic and succinic acids, and a trace of lactic acid. Bendix (1900) reported the occurrence of xylose-fermenting bacteria in the feces of man and cows. According to Salkowski (1900), ordinary protein-decomposing bacteria in a rich nitrogenous medium decompose xylose with the production of the volatile acid, acetic, and the non-volatile acid, succinic. The same worker investigated the behavior of xylose and arabinose when added to putrefying meat, and found that after seven days only traces of the pentoses remained. Volatile acids were formed from both sugars; also a water-insoluble fatty acid from the arabinose.

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[1] This paper covers in part the work submitted to the Graduate School of Yale University by the senior author as part requirement for the degree of Master of Science

The influence of xylose on soil organisms has been studied by several investigators. Stoklasa (1898) showed that denitrification by *B. megatherium*, *B. denitrificans*, *B. subtilis*, and *B. fluorescens-liquefaciens* was at a maximum when xylose was present. Arabinose was found not to be as good a source of energy for the denitrifying bacteria as was xylose. In a later paper (1908) the same author claimed that xylose is superior to all other sugars but arabinose as a source of energy for nitrogen-fixation by Azotobacter. This work was confirmed by Löhnis and Pillai in 1908. Kayser (1894), in the course of an extensive investigation on the production of lactic acid by bacteria obtained from various sources, isolated an organism from sauerkraut which fermented quite a number of sugars, including xylose and arabinose, with the production of lactic acid. Both acetic acid and lactic acid were obtained from glucose, but it is not clearly stated that acetic acid was also formed from the pentoses.

For some time the development of the knowledge of xylose fermentation was associated with studies of spoiled wines. In a series of papers (1894–1901), Gayon and Dubourg reported the isolation from wine of a "mannit-forming" bacterium, an organism capable of producing mannitol from fructose. These investigators found that xylose is the only pentose which is sensitive to the "mannitic ferment." The principle products of the fermentation were acetic and lactic acids, and small quantities of alcohol and carbon dioxide. Mannitol was not formed from the pentose. Mazé and Perrier (1903–1904) published several reports on a "mannite-forming" organism which they had isolated from spoiled wines and which resembled very closely the bacterium described by Gayon and Dubourg. In 1912 Müller-Thurgau and Osterwalder succeeded in isolating four organisms from wine: *B. mannitopoeum*, *Bact. gracile*, *Micrococcus acidivorax* and *Micrococcus variococcus*. They studied the action of these organisms at 25°C. and found that *B. mannitopoeum* attacked both xylose and arabinose, producing more acid from the former than from the latter.

The ratio of acetic to lactic acid was 36:38. *Bact. gracile* and the micrococcus forms did not attack the pentoses. In a later paper (1918) the same authors reported the isolation from wine of two other organisms, *Bact. intermedium* and *Bact. gayoni.* Neither of these attacked arabinose, but both fermented xylose with the production of lactic acid and comparatively large amounts of a volatile acid which was shown to be acetic.

The work of Fred, Peterson and Davenport (1919) initiated a new phase in the development of the knowledge of xylose fermentation. These investigators isolated an apparently new organism from samples of various grains, soils, manure and silage. After a complete study of its cultural, morphological and physiological properties, they named the new form *Lactobacillus pentoaceticus.* They found that acetic and lactic acids were formed, and also a slight trace of ethyl alcohol and carbon dioxide. They studied the effect of various conditions, such as temperature, neutralization with calcium carbonate and sodium hydroxide, anaerobiosis, etc., on the amounts of acid produced. The fermentation of xylose took place rapidly, and within ten to twelve days after inoculation with *L. pentoaceticus* practically all of the sugar was consumed. Periodic determination of the total and volatile acids formed from xylose showed that more than half of the acid was produced from the first to the fourth day, after which the acid production proceeded more slowly. The proportion of the volatile to the non-volatile acid was found to be approximately the same throughout the entire period of fermentation, namely 40 parts of acetic to 60 parts of lactic. The maximum amount of acid per gram of xylose was obtained in cultures containing from 2 to 3 per cent of xylose.

Later work by Anderson, Fred and Peterson (1920) on the pentose-fermenting organism demonstrated that the production of acid from a medium containing 2 per cent xylose is correlated with bacterial viability counts. The rate of acid production was most rapid during the period of maximum growth of the bacteria. Fred, Peterson and Davenport (1920) studied the products formed in the fermentation of various carbohydrates and organic acids by *L. pentoaceticus*, and showed that the hydrolytic action of this organism extends over a wide range of substrates.

Peterson and Fred (1920) and Frazier (1921) investigated the rôle of pentose-fermenting bacteria in the production of corn silage. They found that *L. pentoaceticus* is capable of bringing about decided changes in raw and sterilized corn tissue. When added to raw corn fodder it was able to compete with the fermentation processes which normally occur. In sterilized silage the pentose-fermenting bacteria developed rapidly and produced substances commonly found in good silage, viz., acetic and lactic acids, ethyl alcohol and carbon dioxide. From the standpoint of temperature, oxygen supply and fermentable compounds, silage was found to offer a suitable medium for the growth of the pentose fermenters.[2]

The present investigation is largely in the nature of a corroborative study, and is on the whole confirmatory of the work of Fred and his associates on the biochemical and physiological properties of *Lactobacillus pentoaceticus* first isolated by them. It also includes a brief systematic study of this pentose-destroying organism and its relation to other members of the Lactobacillus group.

EXPERIMENTAL

Isolation and distribution of L. pentoaceticus in nature. At the beginning of our work isolation of xylose-fermenting organisms of this type was attempted by the method described by Fred, Peterson and Davenport (1919) in their original work on the pentoaceticus group. These investigators resorted to the preliminary enrichment method of employing a yeast-water-xylose medium, and then plating on agar. The procedure was found by the writers to be very unsatisfactory, due to the tendency of other organisms, especially molds and yeasts, to overgrow the xylose-fermenters.

The method was modified. One gram of raw material was well shaken in a dilution bottle containing 100 cc. of sterile water. A series of dilutions ranging up to 1:1,000,000 were made and plated out on xylose-yeast-water agar.[3] The plates were incubated at 33°C. for four days under anaerobic conditions, in order to eliminate contaminating molds. Isolation from the lower dilutions was found to be practically impossible, owing to the presence of numerous contaminating organisms other than molds, and very few, if any, Lactobacillus colonies could be obtained when the higher dilutions were employed. The method was repeated several times and abandoned as unsatisfactory.

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[2] The historical review presented above has of necessity been made very brief. For a complete review of the literature on the subject the reader is referred to the senior author's Master of Science essay deposited in the Yale University Library.

The procedure which gave the best results, and the one which was finally adopted in this work, was that used by Kulp and Rettger (1924) in their isolation of *Lactobacillus acidophilus*, by Hunt (1930) in his investigation of high lactic-acid-forming organisms from soils and grains, and by Morishita (1929) in his work on the aciduric organisms found in dental caries. Ten cubic centimeter quantities of yeast-water adjusted to pH 4.2 to 4.8 with acetic acid were dispensed in test tubes. Mineral oil was added to each tube in a quantity sufficient to form a layer about 0.3 cm. deep on the surface of the medium. The tubes were plugged, and sterilized by autoclaving at 15 pounds extra pressure for fifteen minutes. Enough of a 10 per cent xylose solution, previously sterilized by Berkefeld filtration, was then added, under aseptic conditions, to make a concentration in each tube of 0.5 per cent. One cubic centimeter portions of washings of the raw material (1 gram of raw material shaken with 100 cc. sterile water) were added to this medium and the tubes incubated at 33°C. for forty-eight hours. Subcultures were made with the aid of a pipette into tubes of the same medium. After making four or five additional subcultures at intervals of twenty-four hours, the last was plated out on 0.5 per cent xylose yeast-water agar, pH 7.0, and the plates incubated at 33°C. for forty-eight hours.

Examination of the plates showed colonies which appeared to be typical, according to the original description by Fred and his coworkers. These were transferred to 0.5 per cent xylose yeast-water, pH 7.0, incubated for forty-eight hours, and replated in order to make certain of the purity of the culture. Typical colonies were again picked and inoculated into liquid medium. These cultures were subcultured every twenty-four hours during a period of about two weeks, occasional platings being made to determine their purity.

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[3] The yeast water was prepared according to the method used by Hunt and Rettger (1930).

For the determination of volatile and non-volatile acid production, the newly isolated strains were inoculated into Erlenmeyer flasks containing 80 cc. of yeast water to which were added 20 cc. of a 10 per cent solution of xylose sterilized by filtration through a Berkefeld filter. An excess of sterile calcium carbonate was added to each flask, and the cultures incubated for two weeks at 33°C. The method described by Friedemann and Kendall (1929) was used for the estimation of the non-volatile acid. This in-

TABLE 1

Acid produced from xylose by isolated strains

STRAIN	SOURCE	VOLATILE ACID	NON-VOLATILE ACID	TOTAL ACID	RATIO OF VOLATILE TO LACTIC ACID
		grams per 100 cc. of culture	*grams per 100 cc of culture*	*grams per 100 cc of culture*	
PS-1	Sauerkraut	0.7377	1.0163	1.7540	43:57
PS-2	Sauerkraut	0.7202	0.9921	1.7123	42:58
PSI-2	Silage	0.6259	0.8639	1.4898	42:58
PSI-3	Silage	0.7039	0.8593	1.5632	45:55
PSI-4	Silage	0.6268	0.8305	1.4573	43:57
PSO-1	Soil	0.7304	0.8846	1.6150	45:55
PSO-2	Soil	0.7112	0.9823	1.6935	42:58
PSO-3	Soil	0.3630	0.5012	0.8642	42:58
PSO-4	Soil	0.2331	0.2990	0.5321	44:56
PSM-1	Sheep manure	0.6265	0.8653	1.4918	42:58
PHM-1	Horse manure	0.7307	1.0087	1.7394	42:58
PCM-1	Cow manure	0.6515	0.8994	1.5509	42:58
PCM-2	Cow manure	0.7492	0.9524	1.7016	44:56
PCM-3	Cow manure	0.6724	0.8913	1.5637	43:57
PCM-4	Cow manure	0.3561	0.4459	0.8020	44:56
PMM-1	Corn mash	0.1268	?	—	—
	Bran	No isolation	—	—	—
	Rye	No isolation	—	—	—
	Beet meal	No isolation	—	—	—
	Cracked corn	No isolation	—	—	—
	Alfalfa	No isolation	—	—	—
ALF-2	Alfalfa	0.0606	—	—	—

volves the oxidation of lactic acid to acetaldehyde by colloidal manganese dioxide and phosphoric acid. The aldehyde is bound by sodium sulphide added in excess, and the bound acetaldehyde determined by titration with N/10 iodine. The steam distillation and titration method for the determination of volatile acid was employed. The results are given in table 1.

The distribution of this type of xylose fermenters in nature is widespread, as is evidenced by its isolation from various natural sources. Out of twenty-two attempted isolations by the writers sixteen were successful. The distribution of this organism does not seem to be limited to any particular geographical section of the country, for Fred and his associates made their isolations in the mid-western part of the United States.

Sauerkraut, silage, soil and animal manures appear to be excellent sources of Lactobacilli of the pentoaceticus type. Various samples of grains such as corn, alfalfa and bran, which have been shown by various investigators to be good sources of high lactic acid-forming bacteria of the *L. leichmannii* and *L. delbrücki* types, gave no evidence of the presence of the pentose-fermenting group, although these substances are known to contain pentose in some form. No satisfactory explanation for these negative findings can be offered here.

Amount of acid formed from xylose by the type strains.[4] Flasks containing 100 cc. yeast water, 2 grams of Difco xylose and an excess of sterile calcium carbonate were inoculated with type strains P-1, P-2, and P-3 procured from Fred. In this and in all of the subsequent experiments the carbohydrate was sterilized separately by filtration.

After being weighed, each flask was incubated at 33°C. for two weeks. On removal from the incubator the loss in weight of the cultures due to evaporation was made up by adding freshly-boiled distilled water. Aliquot portions were withdrawn and analyzed for volatile and non-volatile acids. The results of the analyses are given in table 2.

The three strains of *L. pentoaceticus* secured from Fred and considered to be typical produced yields of acid lying within the same range characteristic of those isolated by the authors. This fact was assumed to indicate that the recently isolated strains were of the true pentoaceticus type. The ratio of lactic acid to volatile acid was again found to be about 58:42.

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[4] **For list of strains used in this work, and their sources, see table 9.**

TABLE 2

Amount of acid produced by type strains P-1, P-2, and P-3

STRAIN	VOLATILE ACID	NON-VOLATILE ACID	TOTAL ACID	RATIO OF LACTIC TO VOLATILE ACID
	grams per 100 cc. of culture	*grams per 100 cc. of culture*	*grams per 100 cc. of culture*	
P-1	0.7157	0.9885	1.7042	42:58
P-2	0.6862	0.9476	1.6338	42:58
P-3	0.7298	0.9675	1.6973	43:57

Note: In fermentation mixtures to which no neutralizing agent was added the total acid was determined by titration with N/10 sodium hydroxide. When calcium carbonate was present the total acid was determined by combining the volatile and non-volatile acids.

TABLE 3

Rate of acid production from xylose by strain P-1

DAYS	VOLATILE ACID	NON-VOLATILE ACID	TOTAL ACID
	grams per 100 cc. of culture	*grams per 100 cc. of culture*	*grams per 100 cc. of culture*
1	0.1209	0.1603	0.2812
2	0.1954	0.2707	0.4661
3	0.3109	0.3814	0.6923
4	0.3632	0.4992	0.8624
5	0.4183	0.5774	0.9957
6	0.4879	0.6467	1.1346
7	0.5561	0.7185	1.2746
8	0.5867	0.8072	1.3939
10	0.6415	0.8813	1.5228
12	0.6938	0.9197	1.6135
14	0.7089	0.9790	1.6879
16	0.7316	0.9698	1.7014
Uninoculated control. Analyzed every 4 days	0	0	0

Rate of acid production. Strains P-1 and P-2 were inoculated into flasks containing 500 cc. of yeast water to which 6 grams of xylose and an excess of sterile calcium carbonate had been added. The flasks were placed in the incubator at 33°C., and aliquot portions of the contents removed, with aseptic precautions, every twenty-four hours during the first eight days and, following this period, every forty-eight hours. One-tenth cubic centimeter portions from each flask were plated every three days for the purpose of keeping a check on the purity of the cultures. The results of the analyses of the cultures of strain P-1 are given in table 3. Those obtained with strain P-2 are quite similar and are not included here, for the sake of brevity. The rate of acid production is shown graphically in figure 1.

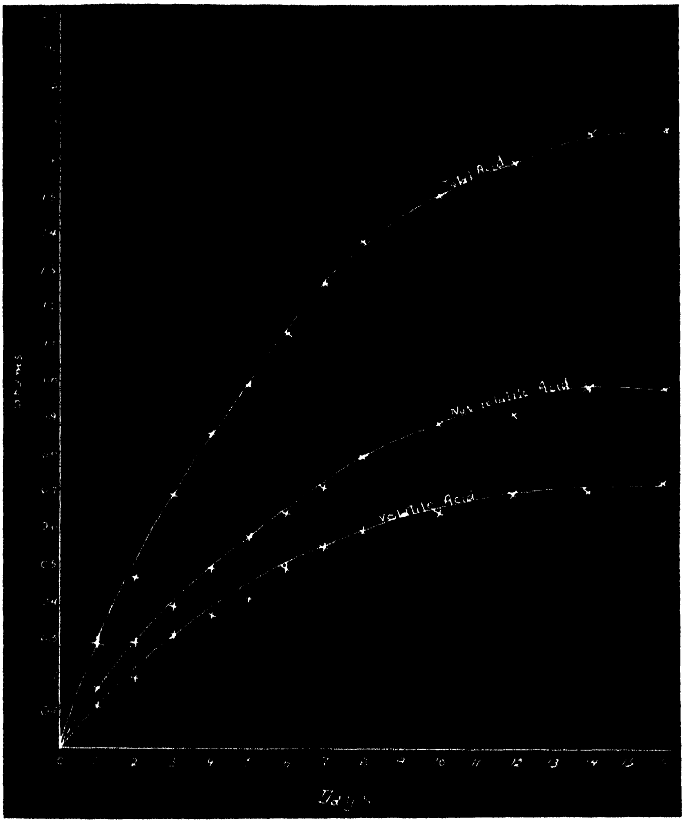

FIG 1. RATE OF ACID PRODUCTION BY STRAIN P-1

Acid production appeared to be most rapid in the first six to eight days, and then to decrease at an almost constant rate until the end of the experiment (fourteen to sixteen days). The acid curve constructed by Fred, Peterson and Davenport (1919), is not as steep as that of the present authors. Fred and his associates, however, did not make their analyses from a single flask, but set up a separate culture for each analysis. As will appear evident from the data presented in this work, there is some variation in the amounts of acid produced by one strain at different times and under practically identical conditions. Therefore, analyses of samples drawn from a single flask probably give a truer picture of what is taking place, in so far as the rate of acid formation is concerned, than analyses of a series of cultures of the same strain. The deviation of several of the values derived from the acid production rate curve cannot be adequately explained. The results obtained in the analyses were checked and found to be correct. The influence of metabolic products, or the presence of uncombined acid, may probably play some part in affecting at times the rate of acid production.

Form of lactic acid produced from xylose by L. pentoaceticus. The rotatory properties of the lactic acid formed by five different strains of the organism from xylose were determined by converting the acid into its zinc salt and making the usual polariscopic observations. The lactic acid was found to be inactive. This finding is in agreement with the observations of Pederson, Peterson, and Fred (1926).

Amount of xylose fermented. Strains P-1, P-2, P-3, PS-1 and PS-2 were inoculated into yeast water containing 2 grams of xylose and an excess of sterile calcium carbonate. After weighing, the flasks were incubated for two weeks at 33°C., following which each flask was made up to original weight and the residual xylose and volatile and non-volatile acids determined. The results are given in table 4.

The ratio of volatile to non-volatile acid was found to be approximately 42:58. From 90 to 95 per cent of the original xylose was destroyed, and from 88 to 92 per cent of the sugar fermented was represented by volatile and non-volatile acids.

These results differ from those obtained by Fred, Peterson and Davenport (1919) in that the percentage of xylose destroyed is higher than the amounts estimated by the previous investigators. However, the proportion of sugar fermented to acids obtained in this work is practically the same as that reported by the above-mentioned authors.

Effect of various conditions on acid production. Up to this point in the present investigation the fermentation of xylose has been studied under conditions described as optimum by previous investigators. It seemed desirable to determine the influence of various conditions, such as temperature, anaerobiosis, etc., on acid production. Such information should be of importance from both the scientific and commercial viewpoints.

TABLE 4

Amounts of xylose fermented in 100 cc. of culture fluid, and acids produced by L. pentoaceticus

STRAIN	ORIGINAL XYLOSE	VOLATILE ACID	NON-VOLATILE ACID	TOTAL ACID	XYLOSE FERMENTED	PER CENT XYLOSE REPRESENTED BY VOLATILE AND NON-VOLATILE ACID	RATIO VOLATILE TO NON-VOLATILE ACID
	grams	*grams*	*grams*	*grams*	*grams*		
P-1	2.0000	0.7157	0.9882	1.7039	1.8564	92	42:58
P-2	2.0000	0.6965	0.9233	1.6198	1.8273	89	43:57
P-3	2.0000	0.7178	0.9769	1.6947	1.8991	89	42:58
PS-1	2.0000	0.7395	1.0211	1.7606	1.9104	92	42:58
PS-2	2.0000	0.7574	0.9641	1.7215	1.9007	90	44:56

Note: The xylose was determined by the method of Youngburg (1927), which involves the conversion of the xylose into furfural by the action of strong phosphoric acid, and colorimetric determination of the furfural.

Effect of neutralization with calcium carbonate on acid production. Strains P-1, P-2, P-3, PS-1 and PS-2 were inoculated into two series of flasks containing yeast water and 2 per cent xylose. To one set an excess of calcium carbonate was added, while the other was left unneutralized. Both series of flasks were incubated at 33°C. for two weeks, and analyzed for volatile and non-volatile acids. The results are shown in table 5.

The cultures to which no calcium carbonate had been added produced only about half as much acid as those which contained an excess of the neutralizing agent. These observations are in harmony with those made by Fred, Peterson and Davenport (1919).

Effect of temperature of incubation on acid production. Strains P-1, P-2, P-3, PS-1 and PS-2 were inoculated into six series of flasks containing xylose yeast water (2 per cent) and an excess of calcium carbonate. One set of cultures was incubated at 10°C., one at 24°C., one at 33°C., one at 37°C. one at 45°C. and one at 55°C. for two weeks. After the incubation period all of the flasks were analyzed for volatile and non-volatile acids. The results are given in table 6.

TABLE 5

Effect of neutralization on acid production

STRAIN	VOLATILE ACID	NON-VOLATILE ACID	TOTAL ACID
A. Cultures neutralized with excess calcium carbonate			
	grams per 100 cc of culture	*grams per 100 cc of culture*	*grams per 100 cc. of culture*
P-1	0.7193	0.9936	1.7129
P-2	0.7006	0.9287	1.6293
P-3	0.7128	0.9836	1.6964
PS-1	0.7491	1.0211	1.7702
PS-2	0.7616	0.9469	1.7085
Uninoculated control	0	0	0
B. Cultures not neutralized			
P-1	0.3438	0.4565	0.8003
P-2	0.3481	0.4623	0.8104
P-3	0.3419	0.4717	0.8136
PS-1	0.3671	0.5070	0.8741
PS-2	0.3665	0.4664	0.8329
Uninoculated control	0	0	0

At 10°C. only slight traces of acid could be detected. A temperature of 33°C. seems to be optimum for the formation of acid by this type of pentose-destroying organisms. At 37°C. only about half as much acid was formed as at 33°C. At first these results were thought to be due to errors in technique, but repeated analyses showed them to be apparently correct. The pentoaceticus type of organisms differs markedly from the granulated thermophilic lactobacilli isolated from grains and cereals, in that 37°C. is the optimum temperature for lactic acid production from glucose by the latter, as compared with 33°C. for the former. At 45°C. only small amounts of acid were produced, and at 55°C. there was no evidence of acid formation. Uninoculated controls at all temperatures gave no acid. The data presented in table 6 are for three strains only. The results obtained with strains PS-1 and PS-2 were practically the same and are not included in the table, for the sake of brevity.

TABLE 6

Effect of temperature of incubation on acid production

TEMPERATURE	VOLATILE ACID	NON-VOLATILE ACID	TOTAL ACID
	Strain P-1		
°C.	*grams per 100 cc. of culture*	*grams per 100 cc of culture*	*grams per 100 cc of culture*
10	Trace	Trace	Trace
24	0 5639	0 7476	1.3115
33	0 7160	0 9886	1 7046
37	0 4107	0.5667	0 9774
45	0 1483	0 1904	0 3387
55	0	0	0
	Strain P-2		
10	Trace	Trace	Trace
24	0 5599	0 6872	1 2471
33	0 7056	0 9353	1 6409
37	0 3280	0 4533	0 7813
45	0 0805	0.0984	0 1789
55	0	0	0
	Strain P-3		
10	Trace	Trace	Trace
24	0 5856	0 8096	1 3952
33	0 7114	0 9821	1.6935
37	0 3702	0 4913	0 8615
45	0 0907	0 1308	0 2215
55	0	0	0

Effect of oxygen removal on acid production. Two series of flasks containing 2 per cent xylose yeast water and an excess of calcium carbonate were inoculated with strains P-1, P-2, P-3, PS-1 and PS-2. One set was incubated under aerobic, and the other under as nearly anaerobic conditions as can be obtained, both at 33°C. The anaerobiosis was effected by alternate exhaustion with a vacuum pump and filling of the anaerobic jar with carbon dioxide. After two weeks incubation the cultures were analyzed for volatile and non-volatile acids. The results of the analyses are given in table 7.

TABLE 7

Influence of oxygen removal on acid production

STRAIN	VOLATILE ACID	NON-VOLATILE ACID	TOTAL ACID
	A. Aerobic cultures		
	grams per 100 cc of culture	*grams per 100 cc of culture*	*grams per 100 cc. of culture*
P-1	0.7463	0.9478	1.6941
P-2	0.6831	0.9426	1.6257
P-3	0.7126	0.9842	1.6968
PS-1	0.7380	1.0214	1.7594
PS-2	0.7401	0.9811	1.7212
Uninoculated control	0	0	0
	B. Anaerobic cultures		
P-1	0.7044	0.9729	1.6773
P-2	0.7173	0.9224	1.6397
P-3	0.6930	0.9569	1.6499
PS-1	0.7419	1.0249	1.7668
PS-2	0.7178	0.9514	1.6692
Uninoculated control	0	0	0

From these data it will be seen that there is no definite relationship between oxygen tension and the amount of acid produced. Differences in the yields of acid lie within the range of variation of individual strains. The evidence obtained here is in harmony with the results secured in a study of the effect on acid production of growth in deep and shallow layers. It was found that the amount of acid produced does not depend on the depth of the medium so long as the culture is kept neutralized by shaking with calcium carbonate.

Influence of composition of medium on acid production. For some time this laboratory has used casein digest[5] as well as yeast water medium for the growth and maintenance of viability of members of the Lactobacillus group. The two media have appeared to possess about equal merit.

In the present investigation a comparison was made of the adaptability of the two media for the production of acid from xylose by the pentose-fermenting lactobacilli. Strains P-1, P-2, P-3, PS-1 and PS-2 were inoculated into two series of flasks, one of which contained yeast water, 2 per cent xylose and an excess of calcium carbonate, and the other the same ingredients with the exception of the yeast water, which was replaced in this series by casein digest. The cultures were incubated at 33°C. for two weeks and analyzed for volatile and non-volatile acids. The results obtained are given in table 8.

TABLE 8

Comparison of the amounts of acid produced in casein digest and in yeast water

STRAIN	VOLATILE ACID	NON-VOLATILE ACID	TOTAL ACID
A. Yeast water medium			
	grams per 100 cc. of culture	*grams per 100 cc of culture*	*grams per 100 cc. of culture*
P-1	0 7136	0 9857	1.6993
P-2	0 6854	0 9464	1.6318
P-3	0 7321	0 9704	1 7025
PS-1	0.8494	0 9003	1.7497
PS-2	0 7186	0 9922	1.7108
Uninoculated control	0	0	0
B. Casein digest medium			
P-1	0 7251	0 8656	1 5907
P-2	0 7396	0 9045	1 6441
P-3	0 7241	0 9572	1 6813
PS-1	0 7355	1 0153	1 7508
PS-2	0 7148	0 9871	1 7019
Uninoculated control	0	0	0

Casein digest and yeast water seem equally well suited as basic media for the growth and physiological activities of *L. pentoaceticus.* The differences in acid production in the two media lie well

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[5] Prepared according to the method of Kulp and Rettger (1924).

TABLE 9

Strains of Lactobacilli used in this work and their sources

From the American Type Culture Collection:	
Del-1.	*L. delbrücki* (899)
Lyc-1.	*L. lycopersici* (4005)
Fer-1.	*L. fermentatae* (4006)
From Fred (University of Wisconsin):	
P-1.	*L. pentoaceticus*
P-2.	*L. pentoaceticus*
P-3.	*L. pcntoaceticus*
Lei-1.	*L. leichmanni* (corn meal)
Lei-2.	*L. leichmanni* (corn meal)
Del-2.	*L. delbrucki*
From Morishita of this laboratory:	
Od-1.	*L. odontolyticus*
Od-2.	*L. odonlolyticus*
Od-3.	*L. odontolyticus*
Od-4.	*L. odontolyticus*
Od-5.	*L. odontolyticus*
Od-6.	*L. odontolyticus*
Authors' isolations:	
PS-1.	*L. pentoaceticus* isolated from sauerkraut
PS-2.	*L. pentoaceticus* isolated from sauerkraut
PSI-2	*L. pentoaceticus* isolated from silage
PSI-3.	*L. pentoaceticus* isolated from silage
PSI-4.	*L. pentoaceticus* isolated from silage
PSO-1.	*L. pentoaceticus* isolated from soil
PSO-2.	*L. pentoaceticus* isolated from soil
PSM-1.	*L. pentoaceticus* isolated from sheep manure
PHM-1.	*L. pentoaceticus* isolated from horse manure
PCM-1.	*L. pentoaceticus* isolated from cow manure
PCM-2.	*L. pentoaceticus* isolated from cow manure
PCM-3.	*L. pentoaceticus* isolated from cow manure

All of the above named lactobacilli were employed in the present biological and chemical study of acid production by members of this genus.

within the range of individual variation of the strains at different times of cultivation.

Comparative study of the morphological, cultural, physiological and serological characteristics of L. pentoaceticus and related aciduric organisms. Fred, Peterson and Davenport (1919) in their original investigation of the pentose-decomposing organism made a study of its morphological, cultural and physiological properties, and on the basis of the results obtained concluded that the bacillus belongs to the Lactobacillus genus. In the present work chief emphasis has been placed on the determination of the position which *L. pentoaceticus* occupies with relation to the aciduric organisms isolated from soil and grains and from cases of dental caries. (See table 9).

Morphology and staining reactions. All of the strains of lactobacilli were inoculated into two sets of tubes containing yeast water and 0.5 per cent xylose. One set was incubated at 37°C. for twenty-four hours, and the other at the same temperature for two weeks. At the end of the respective incubation periods each set was stained by the Gram method and examined microscopically.

L. pentoaceticus was found to be Gram-positive in young culture, whereas in old culture it showed a tendency to be Gram-negative. The organism is a non-sporing, non-capsulated rod, occurring singly, in pairs and in long or short chains. The average measurement was 0.3 to 1μ by 1 to 6μ. In old cultures and in media which were highly acid, forms were observed at times which were as long as 10 to 20μ.

All of the other aciduric organisms examined showed a marked similarity to *L. pentoaceticus* in their morphology and staining reactions, all showing more or less variation within certain rather wide limits.

Colony form. Twenty-four-hour cultures of the different strains were streaked on 0.5 per cent xylose yeast-water agar and incubated for forty-eight hours at 37°C. All of the organisms were found to form small, slightly raised, white or light gray colonies. Those of *L. pentoaceticus* were smooth or "y" type. Strain Fer-1 was found to resemble the pentose-destroying organism in this respect. With the exception of Lyc-1, which formed only "x" or filamentous (rough) colonies, all of the other organisms studied showed the presence of both the "x" and "y" types.

Motility. Very sluggish motility could be detected in eighteen-hour cultures of the pentose-decomposing form. None of the other aciduric types gave any evidence of independent movement.

Growth in 0.5 per cent xylose yeast-water medium. *L. pentoaceticus* was found to produce clouding and sediment in this medium. It was resembled in this respect by all of the other Lactobacilli studied with the exception of Del-1 and Lyc-1, which produced turbidity but no sediment after forty-eight hours' incubation at 37°C.

Gelatin stab. Material scraped from agar plate cultures of the different organisms was inoculated into xylose yeast-water gelatin. No liquefaction of the gelatin was apparent after two weeks' incubation at room temperature. The thermophilic lactic acid bacteria and those of the odontolyticus type resemble *L. pentoaceticus* in giving a beaded, corkscrew-like growth along the line of inoculation.

Growth in agar stabs. All of the strains studied grew well throughout the length of the stab, with the exception of a very small distance below the surface where, although growth was present, it was much smaller in amount than in other parts of the stab. The characteristic beaded growth observed in gelatin was also found in the agar.

Growth in agar shake culture. A heavy inoculum of each type of organism was incorporated into melted xylose (0.5 per cent) yeast-water agar, and well mixed by shaking. The tubes were incubated for one week at 33°C. *L. pentoaceticus* produced a heavy growth throughout the entire medium, but gave no evidence of gas formation. The other aciduric types gave results that were quite similar.

Growth on cooked potato. None of the strains studied gave any visible growth on cooked potato, even after two weeks' incubation at 37°C. and at room temperature.

Action on litmus milk. *L. pentoaceticus* was found to have no action on litmus milk even after two weeks' incubation at 37°C. The thermophilic lactic acid bacteria studied by Hunt (1930) resembled the pentose-fermenting organism in their inability to bring about a change in milk. The strains isolated from cases of dental caries, on the other hand, all produced acid and coagulation of the casein in forty-eight hours.

Indol production and nitrate reduction. Determination of the presence of indol by the Ehrlich-Böhme method, and of nitrites by the use of sulphanilic acid and alpha-naphthylamine, were negative with the pentose-destroying organisms as well as with all of the aciduric strains studied.

Nitrogen utilization. All of the strains were inoculated into culture solutions containing different sources of nitrogen and 0.5 per cent xylose. The media were made perfectly clear, so that growth could be determined easily.

TABLE 10

Nitrogen utilization

STRAIN	0.3 PER CENT BEEF EXTRACT	0.3 PER CENT PEPTONE	0.3 PER CENT GELATIN	0 2 PER CENT ASPARAGINE	0 1 PER CENT UREA	0.2 PER CENT AMMONIUM SULPHATE
P-1	+	+	+	−	−	−
P-2	+	+	+	−	−	−
P-3	+	+	+	−	−	−
PS-1	+	+	+	−	−	−
PS-2	+	+	+	−	−	−
Del-1	+	+	+	−	−	−
Del-2	+	+	+	−	−	−
Lyc-1	+	+	+	−	−	−
Fer-1	+	+	+	−	−	−
Lei-1	+	+	+	−	−	−
Lei-2	+	+	+	−	−	−
LOd-1	+	+	+	−	−	−
LOd-2	+	+	+	−	−	−
LOd-3	+	+	+	−	−	−
LOd-4	+	+	+	−	−	−
LOd-5	+	+	+	−	−	−
LOd-6	+	+	+	−	−	−

No essential differences could be found between *L. pentoaceticus* and the other aciduric forms; media which supported the growth of the pentose-fermenting organism also supported the growth of the other types. The results obtained are presented in table 10.

Fermentation reactions. Yeast water was used as the basic medium in which the fermentation studies were carried out, since it is free from carbohydrates and promotes optimum growth of all the strains. The carbohydates were added in the form of sterile 10 per cent aqueous solutions. All of the fermentable substances used, with the exception of xylose and arabinose, withstood heating in pure aqueous solution for fifteen minutes at 15 pounds steam pressure; the pentoses could be sterilized without injury by filtration only. A change in the hydrogen-ion concentration of the sugar solutions was assumed to indicate that some destructive alteration had taken place.

The inoculum was prepared from a twenty-four-hour yeast-water culture by centrifuging the culture, removing the supernatant fluid and washing the precipitated cells three times with sterile physiological saline solution. A final suspension was prepared having a turbidity of about 8 on the McFarland nephelometer scale. After the making of a purity test, 0.1 cc. of the suspension was used to inoculate the yeast-water sugar media. This method was employed throughout the remainder of this work, except that 1 cc. of washed inoculum was used instead of 0.1 cc. to inoculate the flasks in which the quantitative studies of acid production were made.

The following fermentable substances were used:

Pentoses:
- xylose
- arabinose

Methylpentose:
- rhamnose

Hexoses:
- glucose
- galactose
- mannose
- levulose

Disaccharides:
- sucrose
- maltose
- lactose

Trisaccharides:
- raffinose
- melezitose

Polysaccharides:
- starch
- inulin
- dextrin

Glucosides:
- salicin

Alcohols:
- mannitol
- dulcitol
- inositol
- sorbitol

Milk

Andrade's indicator was employed, and the reactions were read after three and seven days' incubation at 37°C. The results of the fermentation studies are given in table 11.

All of the strains of *L. pentoaceticus* were found to ferment glucose, levulose, mannose, maltose, mannitol, xylose, arabinose, sucrose, salicin and galactose. This pentose-destroying organism resembled all of the other lactobacilli studied here in the inability to break down starch and inulin. The two strains of *L. delbrücki* were found to be similar to *L. pentoaceticus* in every respect but their ability to ferment xylose and the failure of Del-2 to attack salicin.

TABLE 11

Fermentation reactions

STRAIN	GLUCOSE	LEVULOSE	LACTOSE	MANNOSE	MANNITOL	MALTOSE	XYLOSE	ARABINOSE	RHAMNOSE	RAFFINOSE	SUCROSE	DEXTRIN	STARCH	INULIN	SALICIN	DULCITOL	GALACTOSE	INOSITOL	MELEXITOSE	MILK
Del-1	+	+	−	+	+	+	−	−	−	−	+	−	−	−	+	−	+	−	−	−
Del-2	+	+	−	+	+	+	−	−	−	−	+	−	−	−	−	−	+	−	−	−
Lyc-1	+	+	−	−	(+)	+	+	+	−	−	+	−	−	−	−	−	+	−	−	−
Fer-1	+	+	−	−	(+)	+	+	+	−	−	+	−	−	−	−	−	+	−	−	−
Lei-1	+	+	−	+	(+)	+	−	−	−	−	+	+	−	−	−	−	+	−	−	−
Lei-2	+	+	−	+	+	+	−	−	−	−	(+)	+	−	−	−	−	+	−	−	−
P-1	+	+	−	+	+	+	+	+	−	−	+	−	−	−	(+)	−	+	−	−	−
P-2	+	+	−	+	+	+	+	+	−	−	+	−	−	−	(+)	−	+	−	−	−
P-3	+	+	−	+	+	+	+	+	−	−	+	−	−	−	+	−	+	−	−	−
PS-1	+	+	−	+	+	+	+	+	−	−	+	−	−	−	+	−	+	±	−	−
PS-2	+	+	−	+	+	+	+	+	−	−	+	−	−	−	(+)	−	+	−	−	−
Od-1	+	+	+	+	+	+	(+)	+	+	(+)	+	±	−	−	+	−	+	+	+	+
Od-2	+	+	+	+	+	+	(+)	(+)	+	−	+	−	−	−	+	−	+	+	+	+
Od-3	+	+	+	+	−	+	(+)	−	−	+	+	+	−	−	−	−	+	−	−	+
Od-4	+	+	+	+	−	+	(+)	(+)	−	(+)	+	−	−	−	−	−	+	−	+	+
Od-5	+	+	+	+	+	+	(+)	−	−	−	+	±	−	−	+	−	+	(+)	+	+
Od-6	+	+	+	+	+	+	(+)	+	+	−	(+)	−	−	−	+	−	+	+	+	+
Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+, good acid production; (+), slight acid production; −, no acid production; ±, doubtful acid production.

The chief difference between *L. pentoaceticus* and organisms of the *L. odontolyticus* type was the power of the latter to ferment lactose and cause coagulation in milk.

L. leichmanni resembled *L. pentoaceticus* very closely in its fermentation characteristics, with the exception that it was able to ferment dextrin, which the latter could not attack, and that both xylose and arabinose were left unfermented by *L. leichmanni*, whereas *L. pentoaceticus* decomposed them very readily.

L. lycopersici and *L. fermentatae* differed from *L. pentoaceticus* chiefly in their inability to attack mannose and salacin.

From an inspection of the data presented here it seems evident that a strict classification of the Lactobacillus group on the basis of qualitative fermentation reactions is not possible, especially since the same types may vary more or less from time to time in their fermentative properties.

Viability of the pentoaceticus strains in various media. Some difficulty was encountered in this investigation in finding a medium which could be used for maintaining the pentoaceticus strains in stock culture. Early in the investigation it was impossible to preserve their viability for more than a few days in yeast water to which available carbohydrate had been added, or in milk. Different media were used in order to find one which would maintain viability for at least one month without transplanting, namely yeast water, beef peptone bouillon, litmus milk, casein-digest, tomato juice and corn mash. Each of these was used alone, and with the addition of 1 per cent glucose; also, with and without sterile $CaCO_3$. All of the media which contained an excess of $CaCO_3$, with the exception of litmus milk, were capable of maintaining viability for at least one month. The results obtained without the use of $CaCO_3$ were uncertain, a given medium supporting the growth of one strain and not of another. The medium which was finally selected was 1 per cent glucose yeast water to which an excess of sterile $CaCO_3$ had been added. The uncertainty of maintenance of viability in litmus milk has been puzzling, since Peterson and Fred claim that *L. pentoaceticus* remains viable in this medium for periods longer than a month.

Serological relationships of Lactobacillus pentoaceticus and related aciduric organisms. Representative strains of type cultures and newly isolated organisms were selected for serological study. The antigens were prepared as follows: twenty-four-hour-old cultures of the organisms in yeast-water broth containing peptone and glucose were centrifuged, the supernatant fluid removed, and the bacterial cells washed and centrifuged three times in sterile physiological saline solution. The washed cells were suspended in salt solution (turbidity of about 8 on the McFarland nephelometer scale) and a loopful of the suspension plated out to test its purity. Rabbits were immunized with the unheated washed antigens by repeated injections in the marginal ear vein until the agglutinating titer of the serum against the homologous organism was at least 1:800.

The cultures used to prepare the immune sera, and the agglutinating titers of the homologous sera, were as follows:

P-3	1:1600
Lei-1	1:800
Del-1	1:800

Homologous serum for strain Od-3 having a titer of 1:1600 was secured from a collection which had been prepared in this laboratory by Morishita.

Antigen suspensions for the agglutination tests were prepared from all of the strains. Twenty-four-hour cultures of the organisms in yeast water broth containing glucose and peptone were centrifuged and the bacterial sediment washed with physiological saline solution containing 0.25 per cent phenol and having a H-ion concentration of about 7.2. The washing was repeated, with vigorous shaking. By this procedure practically all tendency toward auto-agglutination, which lactobacilli so often manifest, was eliminated. Four dilutions of serum were used, namely 1:100, 1:300, 1:400 and 1:800, and a control containing no serum. Readings were made after twenty-four hours incubation at 37°C. The results of the tests are given in table 12.

Serum Del-1 agglutinated its homologous strain, and Del-2 (which is also *L. delbrucki*). None of the dental organisms or members of the xylose-fermenting group were agglutinated. The agglutination reaction correlates with results obtained in the fermentation studies, in which strains Del-1 and Del-2 gave similar sugar reactions.

Del-1 and Del-2, strains of *L. delbrücki*, appear to be serologically related to the pentoaceticus group as is shown by their agglutination by P-3 antiserum. The fermentation reactions of these organisms were not the same, however; *L. delbrücki* does not ferment xylose, whereas *L. pentoaceticus* attacks this pentose with the production of large amounts of acid. All of the more recently isolated strains of the pentoaceticus group, with the exception of one, PSO-1, were agglutinated by antiserum P-3. Strains PSI-2 and PCM-1 yielded questionable results, because of a tendency toward partial or total auto-agglutination. A comparison of the fermentation and agglutination reactions of the authors' isolations and the type strains secured from Fred can not be made, because data relative to the sugar reactions of the new isolations are not at hand.

TABLE 12

Agglutination reactions—antigenic strains

SERA	Del-1	Del-2	Lyc-1	Fer-1	Lei-1	Lei-2	Od-1	Od-2	Od-3	Od-4	Od-5	Od-6	P-1	P-2
Del-1	++	?	−	−	−	−	−	−	−	−	−	−	−	−
Lei-1	−	−	−	−	++	+	−	−	−	−	−	−	−	−
P-3	++	++	−	−	−	−	−	−	−	−	−	−	++	++
Od-3	−	−	−	−	−	−	+	++	++	+	?	+	−	−

SERA	P-3	PS-2	PSI-2	PSI-3	PSI-4	PSO-1	PSO-2	PSM-1	PHM-1	PCM-1	PCM-2	PCM-3
Del-1	−	−	?	−	−	−	−	−	−	?	−	−
Lei-1	−	−	?	−	−	−	−	−	−	?	−	−
P-3	++	+	?	++	++	−	+	++	+	?	+	+
Od-3	−	−	?	−	−	−	−	−	−	?	−	−

−, no agglutination; ?, some agglutination in control tube; +, agglutination in dilution of 1:100 or 1:200; ++, agglutination in dilution of 1:400 or 1:800.

The only strains agglutinated by Lei-1 antiserum were the homologous organism and another strain of *L. leichmanni*, Lei-2. These results are in close agreement with those obtained in the fermentation reactions of Lei-1 and Lei-2.

The strains isolated from dental caries were the only ones which were agglutinated by antiserum Od-3. The degree of agglutination varied with the different strains. Since the tendency of Od-5 to partial auto-agglutination could not be overcome by the treatment described above, the results with this organism are in three instances designated as doubtful in the table. Perfect correlation between the fermentation and agglutination reactions of members of this group cannot be established.

DISCUSSION AND SUMMARY

Members of the Lactobacillus genus capable of fermenting xylose with the production of large amounts of lactic and acetic acids have been isolated from natural sources and have been shown to be very widespread in nature. No satisfactory attempt has been made here to explain the difficulty or impossibility of isolating xylose-destroying bacteria from certain materials which are known to contain relatively large amounts of pentoses and pentosans. Yields of acids in the ratio of 42 parts of volatile (acetic) to 58 parts of lactic acid have been obtained, but the possibility of securing yields closer to the theoretical 40:60 ratio does not seem very promising, since it has been shown that some of the xylose is utilized by the organisms in their metabolic processes and some is converted to alcohol and carbon dioxide, traces of which may be discovered in the fermentation mixtures.

A study of the morphological, cultural and physiological characteristics of the pentose-destroying organisms studied here leads to the conclusion that they are members of the Lactobacillus genus. These characteristics do not serve, however, to differentiate entirely this species from the types isolated and studied by Hunt and by Morishita. The fermentation reactions were insufficient to indicate sharp differences between *L. pentoaceticus* and organisms like *L. delbrücki*, and *L. leichmanni*. The strains isolated from cases of dental caries also appear to possess the ability of breaking down xylose, but to a much lesser degree than the true pentose-fermenting type.

An attempt was made to establish a relationship between the *L. pentoaceticus* strains and known aciduric organisms of soil and dental origin on a serological basis. Although an antiserum prepared against the xylose-fermenting lactobacilli (pentoaceticus) agglutinated two strains of *L. delbrücki*, it did not react with any other antigens, except those of *L. pentoaceticus*. There appeared to be no serological relationship between the pentoaceticus strains and *L. odontolyticus*, *L. leichmanni*, *L. fermentatae* and *L. lycopersici*. A comparison of the results obtained in the fermentation and agglutination tests did not reveal close correlation between the two types of reactions.

A study of the acid production by *L. pentoaceticus* from xylose has shown that from 88 to 90 per cent of the xylose is destroyed, with the formation of lactic and acetic acids in a ratio of 58:42. The lactic acid produced is inactive. It has been found that an excess of calcium carbonate or some other non-toxic neutralizing agent is necessary to obtain maximum fermentation. Different degrees of oxygen tension do not appear to have any influence on the volume of acid production. The organisms studied have been found to produce different amounts of acids at different temperatures of incubation. This is probably due to the fact that at temperatures other than the optimum the rate of growth and metabolic activity are materially modified.

The field of investigation dealt with in this paper is of interest from both a purely scientific and industrial standpoint. The present work has served to throw further light on a few of the fundamental processes involved in the production of acid from xylose, and has attempted a further classification of the organisms concerned in the process. Much still remains to be done before it can be said that the knowledge of xylose-fermentation by *L. pentoaceticus* and related organisms is complete.

CONCLUSIONS

Pentose-destroying bacteria of the pentoaceticus type are widely distributed in nature.

On a basis of morphological, cultural and fermentation reactions, the different strains of xylose fermenters described in this paper belong to the Lactobacillus genus.

No definite relationships can be established between *L. pentoaceticus* and the known aciduric organisms of soil and dental origin on a basis of ordinary fermentation and agglutination reactions alone.

Xylose is fermented by *L. pentoaceticus* with the formation of volatile(acetic) acid and inactive lactic acid in a ratio of approximately 42:58, the major part of the fermentation taking place in the first week.

A neutralizing agent like calcium carbonate is necessary for the fermentation to go to completion.

The amount of acid produced does not seem to be influenced by the oxygen tension, nor by the use of casein digest instead of yeast water as the basic medium.

From 88 to 90 per cent of the xylose destroyed is represented by volatile and non-volatile acid.

The optimum temperature for the production of acid from xylose by *L. pentoaceticus* is around 33°C.

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FILTRATION TECHNIC

HALDANE GEE

Scripps Institution of Oceanography of the University of California,
La Jolla, California

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Bacteriologists generally must have suffered annoyance on account of mishaps, bacterial and mechanical, with the conventional set-up for Berkefeld filtration. Among the customary difficulties are cracked mantles, leaky stoppers, wet air filters on the suction line, and trouble in aseptically transferring the filtered contents of a suction flask when such is used. Metal foil around the neck of the flask may eliminate dust but it does not keep the stopper air- or liquid- tight.

This writer has had occasion to Berkefeld liquids in lots of several liters for incorporation in culture media. He has been faced also with the problem of eliminating organic activity in samples of sea water for delayed chemical analysis. In this second case it was not permissible to upset the chemical equilibrium in the water by the addition of disinfectants to control bacterial and other biological activity. The successful alternative was the Berkefeld filtration of the water into liter packages satisfactory for storage and shipment.

There has been developed to meet these requirements a modified set-up which other investigators may find of use. The equipment to be described eliminates leaks and minimizes the danger of contamination. It permits the filter to be transferred from one flask or bottle to another with the same degree of safety with which one can inoculate a tube of broth.

APPARATUS

The customary suction flask is abandoned. The filtration is performed directly in 1 liter pyrex Florence flasks. The air filter on the suction line is a separate wide tube plugged with cotton. The neck of the receiving flask is protected by a glass collar. One rubber stopper is used for attaching the filter to the flask and also for holding the collar. The equipment is sterilized in parts in order to prevent the molding of the stopper by the steam pressure into a loose fit for the neck of the flask.

The filters are ordinary Berkefeld candles 15 by 50 mm.

The usual mantle is replaced by a 100 ml. Nessler tube. The Nessler tubes hold more liquid above the filter, which can be operated at full capacity with less attention than in the case of the mantles of conventional size. Nessler tubes also have a thick base which is flat both inside and out, enabling the rubber gaskets to be screwed up tightly without danger of breakage. A hole 10 mm. in diameter is cut in the bottom of the mantle to take the stem of the filter. This can be started with pointed files, using a mixture of camphor and turpentine as a lubricant. The hole is finished with a rattail file similarly lubricated and rotated in the direction opposite to the lead on the teeth spiral. The rubber gaskets are rings cut from pressure tubing. Use one gasket above the mantle and one below. Screw the assembly tight by means of the standard nut.

The filter unit is attached to the receiving flask by means of a carefully selected rubber stopper. This must just enter the neck of the container nicely. A no. 7 stopper is correct for a 1 liter pyrex flask.

The glass sleeve shown in the drawings (fig. 1) is an ordinary thick-walled tumbler with a hole cut in the bottom. This hole must be of such size that the stopper for the flask can be squeezed into it with the top of the stopper just above the outside of the tumbler. The hole can be cut with rattail and half-round files and the camphor and turpentine lubricant. A diameter of 32 mm. is correct for a no. 7 stopper.

The air filter is a pyrex centrifuge tube with an outlet tube sealed to the tip. This must be bent so as to clear the mantle and glass collar when in position.

The mouth of the air filter requires a rubber stopper and delivery for the suction tubing. A two-way glass stopcock is convenient here for controlling the vacuum.

The no. 7 stopper must be neatly bored to take the metal delivery tube of the Berkefeld filter and the exhaust to the air filter. The holes should be so arranged that the parts clear each other, and the neck of the flask and collar, when they are put together. The end of the exhaust tube can be bent to eliminate sucking of the filtrate into the cotton plug.

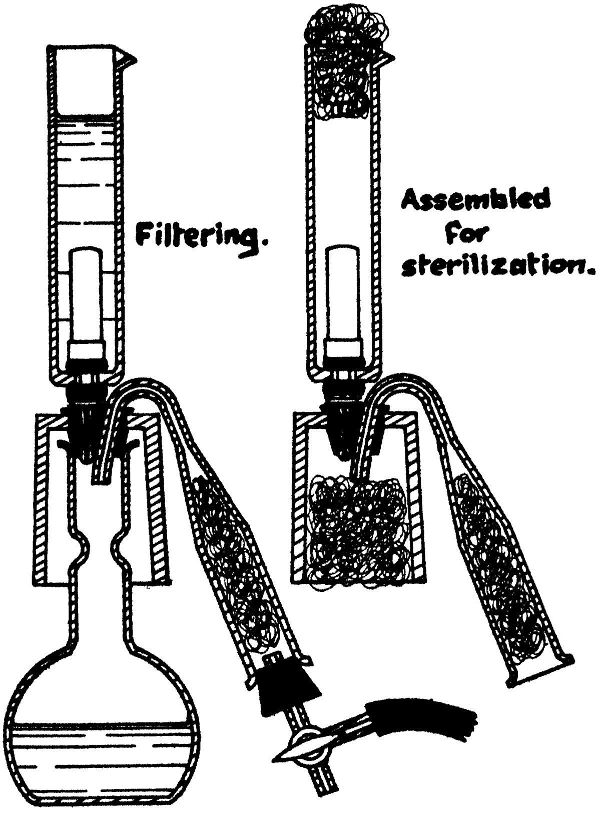

Fig. 1. Berkefeld Set-up: Showing: Left, Special Mantle, Air Filter, and Receptacle Prepared for Sealing; Right, Apparatus Ready for Sterilization

The flasks are prepared in advance with a constriction halfway along the neck for subsequent sealing off.

STERILIZATION

Six flasks are filled at one sterilization of the filter. Prepare these with constrictions, clean, insert well-rolled cotton plugs and tie the necks with paper. Prepare two additional flasks of the same size neck with plugs and caps. Dry sterilize the flasks.

Assemble the filtration unit as shown at the right in the diagram (fig. 1). This should be put together tight and solid. Insert non-absorbent cotton plugs in the top of the mantle, in the air filter, and in the bell of the glass collar. Sterilize in the autoclave at the usual pressure. After sterilization the rubber stopper will be found sealed tight in the collar with swelled flanges above and below. The assembly can be handled safely by means of the collar.

FILTRATION

Remove the paper cap and cotton plug from one of the spare flasks. Remove the plug from the glass collar on the filter unit by means of flamed forceps. Hold the collar in one hand with the first and second fingers over the top of the collar and rubber stopper. Steady the flask with the other hand and seat the stopper firmly. Remove the cotton plug from the top of the mantle. Fill the mantle with liquid. Connect the air filter to the vacuum line and exhaust slowly. The stopper will seal completely as the vacuum is applied.

Run sufficient liquid through the filter to wash it out and to avoid subsequent dilution of the filtrate by water condensed during the autoclaving. Release the vacuum gradually. By making all changes of pressure through the cotton plug contaminating air is not drawn into the flasks. Remove the cap and plug from one of the constricted flasks, and exchange it for the flask containing the first rejected liquid. Filter about 750 ml. of liquid into this flask, keeping the candle covered with liquid. Release the vacuum as before. Change the flasks, using the cotton plug from no. 2 flask for the full flask no. 1. Fill the series of flasks, using the plug from the second spare flask to close the last one containing liquid.

The entire assembly in operation is shown at the left in the diagram (fig. 1).

SEALING

The plugged flasks can be stored for laboratory use if desired. The sterile paper caps can also be transferred one at a time to the flasks along with the cotton plugs, leaving them all plugged and capped.

In the case of the sterile sea water it was necessary to seal the flasks to prevent evaporation and to facilitate shipment. For this purpose they were wired onto an improvised potter's wheel, in this case a vertical kymograph drum. The sealing was accomplished with a small oxyacetylene flame. One operator spins the wheel and draws out the neck above the constriction by means of a pair of tongs. The second operator manipulates the torch. It is of course necessary to rotate the flask with care in order to prevent splashing the liquid on the hot seal, probably with cracking and possibly with explosion. The potter's wheel arrangement eliminates these dangers. The cotton plugs are left in place during the operation and the sealing can be completed before they catch fire.

USE OF BOTTLES

Pyrex 1 liter bottles, also, were used by the author for storage and shipment of Berkefeld filtrates. The set-up is similar. In adapting the device to bottles of this size, no. 6 rubber stoppers should be used. The glass collar must be shorter to clear the shoulders of the bottles. A small cup 40 mm. high and 60 mm. diameter was used. The whole was cut in the bottom to a diameter of 28 mm. The no. 6 stopper when wet can be pushed almost through the collar, and remains tight with the filter and suction tube in place after sterilization. The small end of the stopper will just make a secure fit on the neck of the bottle.

Clean the bottles and provide them with well-rolled cotton plugs and paper caps. Number the glass stoppers to prevent interchange, and place them in the tops of short cylinders. Cover the tops of the cylinders with cotton pads folded in cheesecloth. Tie the pads down. Dry sterilize the bottles and glass stoppers. A dummy bottle can be used for washing the filter as in the case of the liter flasks.

When the first bottle has been filled, remove the filter and collar, and attach to the second bottle. Lift the stopper for the first bottle from its cylinder by means of the cotton pad on top. Seat the stopper firmly in the neck of the bottle, holding it by the same sterile pad. Tie the pad down, using a hitch to prevent slipping of the string.

Precautions already mentioned should be observed, with regard to keeping the candle covered with liquid, and gradual release of the vacuum through the stopcock before changing bottles.

These packages have been shipped 10,000 miles as rail freight and deck cargo without damage or leakage. The sealed flasks showed no mold after one year. Molds were occasionally recovered from the bottles. The sealed flasks are less convenient than the bottles, but more certain bacteriologically.

SUMMARY

An improved filtering device is described for use in connection with Berkefeld operations.

MECHANICAL SPINNER FOR ESMARCH CULTURES

HALDANE GEE

Scripps Institution of Oceanography of the University of California, La Jolla, California

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Shortly after Koch's plating methods were introduced, there was developed by Esmarch (1886) a procedure for spreading the solid films inside tubes. The Esmarch roll tube culture is familiar to bacteriologists, although it does not appear to have found general favor. In recent years the method was placed on a strictly quantitative basis by Wilson (1922), who found it advantageous in elaborate counts for statistical work.

Roll-tube cultures are more convenient than plate cultures for many purposes. It is possible that they would be more widely used if they required less manual dexterity and practice in preparation, and were suitable for the variety of media which can be used in the regulation plating method. In order to be incubated, examined, and counted satisfactorily, the film must be hard, adhering to the tube and free from wrinkles; it should not be thick at the base of the tube, and should not splash on the cotton plug. Esmarch developed the technic primarily for gelatin. Wilson used a hard, 2.5 per cent agar, which involves a high inoculating temperature.

For critical studies of marine organisms at this laboratory it was necessary to make viable counts at sea, preferably by a procedure permitting subsequent transfer of colonies. Petri dish cultures cannot be handled conveniently on board ship. Some have used tables hung on gimbals, similar to a compass mounting, for the solidification, but this arrangement is a makeshift.

The roll tube looked like a logical solution of this difficulty. It is possible that Esmarch had the same problem in mind when he introduced the method. After describing the preparation of the roll tubes in detail, he remarks that he used it to advantage while on an eight-day cruise of the North Sea.

This author, however, has not been satisfied with different attempts to prepare hand-rolled tubes. The usual causes of failure are wrinkling of the films at the moment of solidification, and subsequent sliding of the medium down the tube during incubation. It is possible that either the higher summer temperatures of this continent or the difficulty of controlling artificial heating during the winter prejudices the chances for success.

It was found that most of the difficulties can be eliminated by preparing the tubes in a centrifugal machine. The device described in this paper turns out perfect films which can be solidified promptly and inverted in the incubator. It worked satisfactorily on a small boat rolling and pitching on the Pacific swells. The essentials of the machine are set forth here for the special information of bacteriologists interested in hydrobiology and for the general comment of the science. Wider applications suggest themselves to which reference will be made later.

APPARATUS

The mechanical arrangements are shown diagrammatically in figure 1. A culture tube containing liquified and inoculated medium is spun vertically in a water bath by means of a motor. As soon as the film has climbed the tube, ice water is run into the bath, freezing the medium in a few moments. The tube can then be removed and incubated.

The sizes given were found convenient for the present work on board ship involving counting and transfer of the colonies. The instrument may be considered as still in the developmental stage and subject to change and improvement. Thin-walled Pyrex tubes 25 by 200 mm. were used for the cultures. The water bath was a cylinder 10 by 20 cm., attached to a baseboard which could be moved vertically and clamped in any position. A small ball-bearing was cemented horizontally in the center of the bottom of the water bath with plaster of Paris. The bearing used for the 25 mm. tubes was taken from an automobile

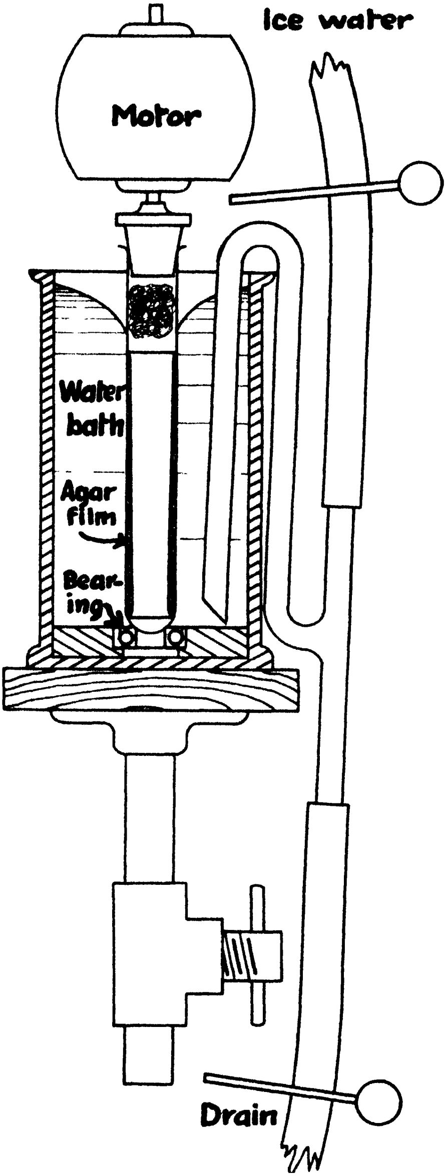

FIG. 1. SPINNER SHOWING DISTRIBUTION OF AGAR INSIDE TUBE AT THE INSTANT THE FILM IS CONGEALED

generator with $\frac{5}{8}$-inch shaft. The water bath was fitted with a wide syphon extending to the bottom and bent close to the wall in order to be clear of the revolving tube. The spinning was accomplished with an automobile horn motor. No gear reduction was necessary. The motor was mounted so that it could be centered over the ball step bearing. The tubes were driven by means of a rubber stopper attached to the shaft of the motor. The stopper was fastened to a collar on the shaft with pins. When in place it was filed true to the axis of rotation with the motor in operation. The motor current was supplied by a 6-volt storage battery. This was placed in series with a sliding resistance made of six strands of 22 gauge nichrome wire in parallel about 3 feet long and twisted together. The 6-volt outfit was found convenient for work in the laboratory also. It was found to be more substantial and to possess a higher starting torque than small 110-volt motors which were tried out. An ordinary automobile battery can supply current for preparing several hundred tubes. The syphon in the water bath was connected with an overhead reservoir of ice water and also to a drain.

MEDIA

Either gelatin or agar may be used in the medium. Nutrient broth containing 10 per cent gelatin was found satisfactory. Gelatin sticks immediately to the inside of the tube, but it must be incubated well below its melting point to prevent softening and collapse. Broth containing 1.5 per cent agar was also found satisfactory where high incubation temperatures were necessary. The agar must be carefully handled in order to prevent sliding down the tube.

OPERATION

Plug the tubes for rolling tightly and dry sterilize them. To prepare a culture, melt sufficient medium for the work, and pour 15 ml. into each of the roll tubes. Hold them at 30°C. in the case of gelatin, and at 45°C. in the case of 1.5 per cent agar. It is advisable to immerse the tubes almost up to the neck and to hold at the proper temperature for fifteen minutes. Have ice water ready for the water bath. Inoculate a tube prepared with medium by dropping in 1 ml. of culture, suspension or dilution. Place the tube in the spinner, seating the stopper first and then raising the base so that the lower end rests firmly in the bearing but without introducing too much friction. The tube

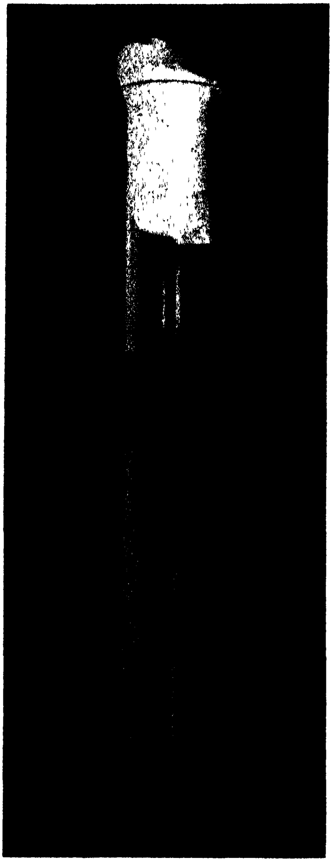

FIG 2 PHOTOGRAPH OF MECHANICALLY PREPARED CULTURE OF MARINE ORGANISMS ON AN AGAR MEDIUM

Shows uniform distribution of colonies, facilitating counting Procedure removes medium and inoculant from the base of the tube.

should rotate freely but without wobble. Start the motor and increase its speed until the medium climbs near the plug. The rise of the medium can be watched through the outside. Fill the bath with ice water and maintain the speed of the motor for a minute. The motor can then be stopped and the tube removed. It should show a clear, uniform film, adhering to the glass and rising to within a safe distance of the cotton plug. The bottom of the tube should be empty.

Gelatin tubes may be incubated upside down immediately. It is advisable to lay agar tubes horizontal in the incubator for several hours before inverting.

No advantage was found in the use of agar stiffer than 1.5 per cent. The best results were obtained by holding agar medium of this concentration at 45°C. for fifteen minutes after melting and before spinning and inoculating. Stiffer agar is not an advantage in preventing the films from sliding, and it requires a warm water bath for the spinning operation unless the temperature of inoculation is made unreasonably high.

Esmarch used gum arabic and fish glue in his agar films. In the present work the agar was prepared in the conventional way, and it was found that the tubes could be inverted without collapse if they were held horizontally for a few hours after the rolling. It is possible that agar-agar will prove more satisfactory than purified Bacto-agar, since it contains a higher percentage of salts,—such as calcium which seems to be involved in the solidification.

The attempt was then made to incorporate both agar and gelatin in a single medium, but the mixture is less satisfactory than either constituent alone unless the proper physico-chemical treatment to prevent the formation of a mush is discovered. Recent research in photographic materials may furnish a satisfactory combination of the two substances.

APPLICATIONS

The 25 mm. tubes were used to facilitate picking of the colonies. A similar mechanical spinner could be designed for smaller tubes exclusively for counting purposes. It may be found convenient to use tubes 15 by 200 mm., thus cutting down the initial cost and incubator space but retaining sufficient length to prevent splashing of the medium on the plugs.

The apparatus as here described is admittedly crude. If additional uses for roll tubes occur to bacteriologists, there are several refinements which suggest themselves. The lower bearing could be held in a spring mounting to prevent occasional sticking and changes of speed. There could also be substituted for it a lower shaft operating through a stuffing gland at the same speed as the upper shaft. A tachometer on the motor would permit the spinning of the medium to a standard height without observation of the climbing film. The water bath could be provided with a copper coil joined to a two-way supply, with temperature indicated on a dial thermometer and controlled by a mixing fixture supplied with hot water and ice water. In this way the bath could be changed promptly from warm water for the spinning operation to cold for the freezing.

A fully mechanical arrangement could be devised for viable counts. This may be of interest in sterilizer control and in the milk and fermentation industries. Using a mechanical spinner operated in a sterile chamber, it is possible to visualize a mechanical device withdrawing samples at intervals, spinning tubes with or without dilution of the inoculant, and storing them in a sterile incubator for examination by a routine worker.

SUMMARY

A mechanical device is described for preparing Esmarch roll tubes.

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THE REDUCTION OF SULPHUR CONTAINING COMPOUNDS IN WOOD PULP AND PAPER MANUFACTURE

T. D. BECKWITH AND J. R. MOSER

Department of Bacteriology, University of California, Berkeley, California

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It is the practice in the pulp and paper industry to transport the comminuted wood fibre throughout the plant where manufacture takes place by means of water. The various channels through which this water circulates comprise what is in part a closed system. Within such a system, different varieties of accumulation are likely to make their appearance and these growths constitute a cause of decided decrease in efficiency.

Upon the inside of certain conducting pipes, gelatinous coatings tend to form. This zoogloeal mass becomes blackened. After a time, portions of it break away, and mix with the suspended wood fibre which thus becomes darkened. The resulting paper will be tinted from a light to a dark grey and much of it must be discarded. This discussion deals with the causes underlying this blackening of wood pulp.

There have been advanced a number of hypotheses explaining this difficulty. One of the more widely accepted explanations states that iron from the piping is changed chemically and that, thus, the only remedy lies in replacing iron conduits with those of other materials such as cement. The difficulty with this statement lies in the observation that these black accumulations occur not only in contact with iron but also upon other surfaces such as wood and cement where there is no iron. Moreover, microscopic examination of this blackened material does not reveal the presence of any of the so-called iron bacteria. Another idea advanced to deal with explanation of this change is that tannin is split off from certain combinations in the wood and that this in turn unites with iron to produce the dark compound. This statement also seems to require further substantiation.

It will be recalled that Beijerinck (1895) proved that there are certain vibrios which produce hydrogen sulphide anaerobically found in the mud of certain streams. With traces of iron present, black iron sulphide results. Van Delden (1904) later succeeded in isolating these forms in pure culture. More recently Bastin (1926) has shown that these organisms are related to the destruction of sulphates in ground waters adjacent to oil wells with consequent effect upon the oil. It seemed possible that this organism, denominated *Microspora desulphuricans* by Beijerinck might be instrumental in producing the change in wood pulp and it was decided to examine this hypothesis.

If *Microspora desulphuricans* is active as an organism producing blackening of wood pulp, then certain materials necessary for its metabolism must be available. Primarily there must be sulphur-bearing materials, notably sulphates, sulphites or sulphur-containing organic compounds. Iron must be available to produce iron sulphide subsequent to the production of hydrogen sulphide.

These necessities are present and available in this environment. A certain amount of sulphate is used in purification of the water utilized. In addition, there is a small amount of sulphate naturally present in the water. The largest portion available however is derived from the wood itself, since repeated examinations by chemists of the industry have indicated from 0.12 to 20.0 per cent SO_3 content of the ash of pulp in various stages of manufacture. Sulphite, also, is used in quantity in the pulp and paper industry as a bleach and thus is present here. Wood also contains sulphur, organically combined in amino acid combination as well as in other complexes. Iron likewise is ready to be utilized in white water which is the suspension of pulp in water; for examination of the ash from wood taken from various sources gives evidence of an iron content varying between 0.87 and 3.67 per cent.

Theoretically, therefore, it should be possible to show that an agent which may bring about blackening of pulp is this form which originally was described by Beijerinck from the muds of Holland. Therefore, we prepared the various media as laid down by Beijerinck, by Van Delden and by Bastin for reduction of sulphates and sulphites according to these formulae:

Beijerinck's alkaline medium

Dibasic potassium phosphate	0.5 gram
Asparagin	1.0 gram
Magnesium sulphate	2.5 grams
Sodium lactate	5.0 grams
Sodium carbonate	1.0 gram
Ferrous ammonium sulphate	0.5 gram
Tap water	1,000 cc.

Van Delden's sulphite medium

Dibasic potassium phosphate	0.5 gram
Asparagin	1.0 gram
Magnesium sulphate	2.5 grams
Sodium lactate	2.0 grams
Sodium sulphite (7 H_2O)	0.5 gram
Ferrous ammonium sulphate	0.5 gram
Tap water	1,000 cc.

Bastin's medium

Dibasic potassium phosphate	0.5 gram
Asparagin	1.0 gram
Magnesium sulphate	2.5 grams
Sodium lactate	5.0 grams
Ferrous ammonium sulphate	Trace
Distilled water	1,000 cc.

Through the courtesy of the Great Western Electro-Chemical Company, a series of samples of the black accumulation which it was desired to examine were obtained from pulp mills in Washington. There were eight of these and they were taken from various points about the plants where this undesired material had appeared. These were now examined as follows.

Microscopic smears made according to the Gram method show a mass of organisms which are predominantly negative to this reaction. In shape they are somewhat pleomorphic and it is indicative that they are somewhat curved. Mixed with them are hyphae of certain fungi. A few conidiospores of some species of Fusarium may be seen.

The various media mentioned above were made up, both with and without agar, then inoculated with small amounts of this black material to produce deep cultures and these were layered with a sterile mixture of paraffin and vaseline to maintain anaerobic oxygen tension. Two series of cultures were thus prepared and of these one was incubated at 37°C. while the other was placed at 28°C. The incubation period was five days in all instances. All preparations have been made in duplicate.

Results were obtained from these cultures as follows. The formula of Beijerinck made up without addition of agar gave a positive black growth with two of the eight specimens at each of the two temperatures utilized for incubation. When this particular fluid was rendered semi-solid with agar, there were four positives at 37°C. and two at 28°C. With the Van Delden formula positive blackening was obtained from all of the eight specimens, both with and without agar, and at each of the incubation temperatures. Bastin fluid both by itself and with agar added gave positive results in all instances at 37°C. but there were developed only six out of eight showing positive blackening when the incubation was at 28°C.

The results of this experimentation carried on thus far indicates therefore that sulphate and sulphite are readily reduced by bacteria present within the samples under consideration and that the production of hydrogen sulphide in contact with iron compounds results in the formation of iron sulphide. Further evidence of this reaction was furnished by the fact that during the period of incubation, readily discernible quantities of hydrogen sulphide were present in the atmosphere of the incubators. Control tests made by pouring plates of the agar media and then streaking them with the materials gave consistently negative results and thus it was shown that this change takes place only under anaerobic conditions.

The most favorable results were obtained through use of the Van Delden formula which contains both sulphate and sulphite. At this point it was desired to determine whether the organisms present in these black materials can act upon sulphate by itself. The Bastin formula had proven to be more favorable in indicating reduction of sulphate only than had that of Beijerinck but nevertheless it seemed possible that even better results might be obtained through certain alterations in this last named medium. The formula which was finally adopted to indicate sulphate reduction was therefore a modification of Bastin's. The amount of sodium lactate was cut down and ferrous chloride was substituted for ferrous ammonium sulphate. As finally constituted, it was as follows:

Medium for sulphate reduction

Dibasic potassium phosphate	0 5 gram
Asparagin	1 0 gram
Magnesium sulphate	2 5 grams
Sodium lactate	2 0 grams
Ferrous chloride	0 1 gram
Tap water	1,000 cc.
Agar	20 0 grams

By use of this formula, positive results were obtained at 37°C. incubation in from three to five days with all of the eight samples. It was thus proven that there are organisms here which are able to break down sulphate with final formation of iron sulphide.

Attention was now given to the possible ability of the bacterial flora of the materials in question to reduce sulphite with no other source of sulphur present. For this purpose a medium was elaborated according to the formula which follows:

Medium for sulphite reduction

Dibasic potassium phosphate	0 5 gram
Magnesium chloride	2 5 grams
Sodium sulphite	1 0 gram
Ferric chloride	0 1 gram
Asparagin	1.0 gram
Sodium lactate	2 0 grams
Tap water	1,000 cc.
Agar	20 0 grams

By means of the medium thus prepared inoculations were made as outlined in the previous discussion and with anaerobic technique. Again, all incubations were carried through both at 37°C. and at 28°C. As before, all work was set up in duplicate. A parallel series made from the same materials using this medium under aerobic conditions in Petri dishes gave again consistently negative results but the attempts to demonstrate the formation of iron sulphide in the anaerobic preparations were successful. Definite black zones of growth appeared in all of the tubes within a five-day period of incubation and thus it became evident that these forms within the black aggregations appearing in white-water conducting-channels contain organisms which have the power to reduce sulphite with formation of hydrogen sulphide as indicated by odor and with subsequent appearance of iron sulphide.

The next step in logical sequence was to determine whether these organisms with which we have been dealing may be able to break down other matter, organic in character but which contains sulphur within its structure. A number of attempts were made with various combinations of materials in different formulae. It was finally determined that the absence of carbohydrate is a favorable consideration and that there is sufficient sulphur in peptone for the purpose in view. The formula as finally adopted contains the following materials:

Medium for reduction of organic sulphur

Peptone (Difco)	20 0 grams
Ferric chloride	0 1 gram
Tap water	1,000 cc.
Agar	20.0 grams

With this medium, aerobic methods gave again results entirely negative in character but with deep agar preparations with seal and 37°C. incubation for a five-day period, all eight samples under examination produced hydrogen sulphide and all of these tubes made in duplicate showed a heavy zone of black growth. It thus becomes evident that these forms may reduce organic sulphur-containing compounds in addition to sulphates and sulphites.

Following the series of experiments outlined above, attention was given to the question of the necessary chemical condition of the iron entering into this reaction. These media were made up with ferric iron in one series and with ferrous chloride in the second series. The blackening appeared to take place with equal readiness with the iron in either condition. Thus, either ferrous or ferric iron may be utilized.

In work in the field of water bacteriology it is well recognized that a small content of manganese may give rise to black stains caused by manganic compounds which are produced. It was deemed likely that a similar change might be proven by bacteriologic methods to take place with organisms derived from these samples containing the iron sulphide producing organisms. In case a manganic compound were to be formed, a dark zone should be produced. On the other hand, the production of a manganous compound as manganous sulphide would give a pink color. For determining the effect of reduction of sulphate and subsequent reaction upon manganese, the following formula was elaborated as being efficient.

Sulphate manganese medium

Dibasic potassium phosphate	0 5 gram
Magnesium sulphate	2 5 grams
Manganous chloride	0 1 gram
Asparagin	1 0 gram
Sodium lactate	2 0 grams
Tap water	1,000 cc.
Agar	20 0 grams

With this medium each of the samples in duplicate preparation, and with anaerobic technique, produced during a five-day period of incubation at 37°C. pink colonies indicating thus the formation of manganous sulphide.

Sulphite likewise with manganous chloride undergoes a similar set of reactions. The following medium was compounded.

Sulphite manganese medium

Dibasic potassium phosphate	0 5 gram
Sodium sulphite	1 0 gram
Manganous chloride	0.1 gram
Asparagin	1 0 gram
Sodium lactate	2.0 grams
Tap water	1,000 cc.
Agar	20.0 grams

With this medium and following the conditions outlined in the preceding paragraph, seven of the eight specimens of black material under test gave definite pink colonies so that it was indicated that these organisms may interact likewise with sulphite and a manganous salt. With these, manganous sulphide is produced.

Following the above series of experiments, attention was given to isolation of various strains of the organism which produces these changes. For this purpose we have used materials from various sources including the black accumulations from pulp mills in Washington noted in the earlier part of this discussion, Berkeley sewage just beyond its outfall into San Francisco Bay, black San Francisco Bay muds where the water is brackish and crude sewage from Los Angeles. We have used the sulphite-iron chloride medium mentioned previously in all instances for this purpose and have succeeded in the isolation of eight different strains. We have repeated the original observations of Beijerinck (1895) in this work for it is to be noted particularly that in close connection with the Microspora are to be found various strains of a colon-like organism. Repeated attempts at purification thus become necessary if one is to obtain the sulphur-reducing organism free of this aerobic form which lives in close connection with it under natural conditions. In many instances, five or six generations of deep colony formation will pass before one may obtain a strain which will not show aerobic contamination when tested appropriately. The various strains which we finally succeeded in obtaining as pure cultures were now examined as to certain of their cultural reactions. All of them proved to be non-motile, distinctly curved vibrios which gave a Gram-negative stain and the characteristics of the eight strains in other regards have been incorporated in table 1.

The sources of these strains were the following:

1. Los Angeles sewage
2. White water from pulp mill
3. White water from pulp mill
4. White water from pulp mill
5. Berkeley sewage outfall
6. Los Angeles sewage
7. Cardboard mill
8. Berkeley sewage outfall

The above reactions were read after five days' incubation at 37°C. It should be stated that the amount of gas given off in the various fermentation reactions was small in all instances as observed in the Durham tube. Indol was determined from tryptophane broth by addition of Salkowski reagents. It is noteworthy that marked variation was indicated with certain of the sugars, particularly lactose. We have observed that it is wise to transplant cultures growing in the sulphite agar which has been described during the course of this discussion after approximately a two-week period. The various observations which have been included in this work dealing with inoculations of the black materials from pulp mills in sulphate, sulphite and organic sulphur containing media are also all confirmed when the various strains of the purified organisms are placed within them. In other words, blackening takes place in all instances within five days at 37°C.

TABLE 1

Cultural reactions of eight strains of Microspora desulphuricans

	STRAIN 1	STRAIN 2	STRAIN 3	STRAIN 4	STRAIN 5	STRAIN 6	STRAIN 7	STRAIN 8
Gelatin liquefaction	−	−	−	−	−	−	−	−
Nitrate reduction	+	+	+	+	+	+	+	+
Indol	+	+	+	+	+	+	+	+
Litmus milk	*, †	*	*, †	*	*, †	*	*	*
Acetyl-methyl-carbinol	−	−	−	−	−	−	−	−
Methyl red	+	+	+	+	+	+	+	+
Glucose	×	×	+	×	+	×	×	×
Galactose	×	×	×	×	×	+	+	×
Lactose	−	+	×	+	−	−	+	−
Sucrose	+	+	+	+	+	+	+	+
Maltose	−	−	+	−	+	−	−	−
Levulose	−	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−	−	−
Mannitol	×	×	×	×	×	×	+	×
Glycerol	−	−	−	−	−	−	−	−
Dextrin	−	−	−	−	−	−	−	−
Salicin	−	+	+	−	−	+	−	−

* Decolorized. † Coagulated. *Note:* + in first six lines means positive reaction. In other lines, acid without evident gas. × = acid and gas.

The source of these forms in the pulp and paper industry will be found to be the water supply used within the plant. If one centrifugalizes 50 cc. of the water taken from such a source at high speed for thirty to sixty minutes, one will obtain a small amount of sediment. In many instances, if a portion of this precipitated material be added to the sulphite medium which has been described, darkening due to the formation of iron sulphide together with formation of hydrogen sulphide will appear as incubation progresses.

SUMMARY

Blackening during the course of manufacture of pulp stock is due to the formation of black accumulations in and upon the equipment used in the industry. This growth is bacterial in origin. The organism in question is *Microspora desulphuricans*. This reduces sulphur-containing compounds with production of hydrogen sulphide which in turn unites with iron to form sulphide. Both the sulphur and the iron necessary to produce this change are present in the wood used in manufacture of the product but it is possible that additional sulphur may be utilized from sulphite used in the processes. Iron from iron piping may also aid the change. The source of the organisms is the water supply. Cultural reactions of eight pure strains of the organism have been carried out. In certain carbohydrate reactions, these are found to be variable.

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UNIFORM GROWTH AND PROGRESSION OF MOBILE COLONIES OF BACTERIA IN LIQUID PLATES

L B BIBB

Major, Medical Corps, U S Army

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In previous papers (Bibb (1925, 1927, 1927-28)) mobile colonies in tubes have been described. The experiments here reported deal with the gross appearance of mobile colonies grown in liquid media, contained not in tubes but in thin, horizontal plates. *Salmonella schottmuelleri* and an unidentified organism obtained from the mouth were studied.

TECHNIC

Plain meat infusion broth was used, of pH 7.6. This medium was held by capillarity between two panes of glass supported in the horizontal position. They were rinsed, dried and then assembled in pairs. The two panes of glass constituting a single plate were held apart by small pieces of wooden applicator fixed by adhesive plaster near the corners. The plates were wrapped in paper and autoclaved. The paper was removed, and the plates were placed on a rack with a camera focused on them from above. The apparatus was housed in a basement with a concrete floor. The medium was run in through a capillary pipette and allowed to rest until all currents had subsided. Inoculation was done through capillary pipettes which were often left *in situ* between the plates. An electric light was on the floor beneath and slightly to one side of the plates. The cultures were grown at room temperature in an almost dark room, and photographed without being disturbed.

MOBILE RING COLONIES

Figures 1 and 2 show a mobile colony at two different stages. Figure 1 shows the colony two hours and thirty-eight minutes after inoculation. The inoculum consisted of organisms taken from a plain broth culture only a few hours old and transferred with the least possible disturbance or dilution, so as to avoid lag. Although two mobile colonies had been often seen, one behind the other, in the motility tubes previously described, (Bibb (1927–28)) it was nevertheless a surprise to find two expanding rings, one within the other. Figure 2 shows this colony a little over six hours old. This organism was obtained from the throat of a laboratory assistant. It was not identified. Nevertheless, the mobile colonies of certain strains of *Salmonella schottmuelleri* were so similar to this that the author believes this colony is representative of vigorous motile organisms of the intestinal group. One of the points of particular interest in this colony is the peripheral ring. This seems compounded of three parts. Within this ring is a zone of relative vacuity or emptiness. Then, not very far from the center and best seen in figure 1, is another dense ring of bacteria, not triple-layered, but apparently of homogeneous consistency. This ring is not isolated like the

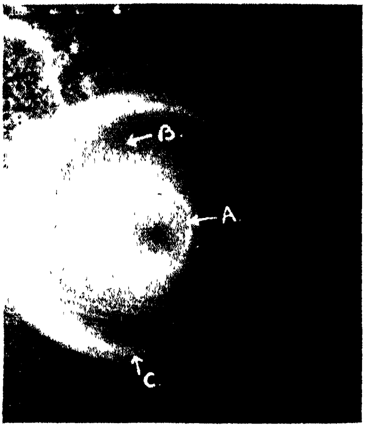

FIG. 1. MOBILE COLONY WITH MULTIPLE EXPANDING CONCENTRIC RINGS

Grown in liquid media in horizontal plate. Age of colony two hours thirty-eight minutes Note the relatively clear space *B* between the dense inner ring *A* and the peculiar peripheral ring *C* *A* and *C* are both expansile *C* has three layers that is a clear space between two dense white rings. The next figure shows the colony four hours later (two-thirds natural size) peripheral ring, but lies in the midst of a turbid layer or sheet of bacteria, from which it detaches itself by its density. In figure 2, there is an incomplete triple-layered ring lying between the peripheral ring and the homogeneous ring just described. The middle of the bull's eye colony shows a circular area of fairly smooth, homogeneous turbidity.

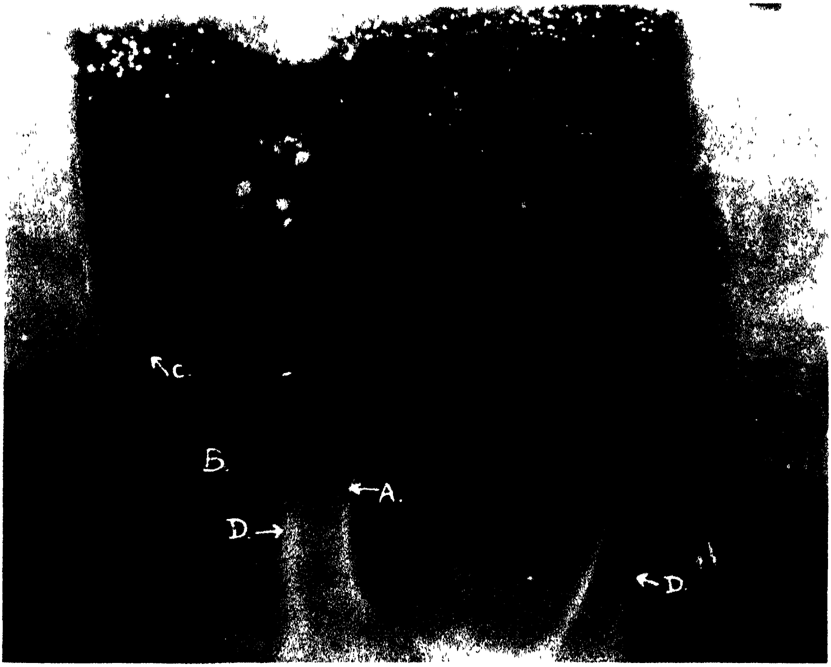

FIG 2. SAME COLONY AS FIGURE 1

Age six hours fifteen minutes. Both peripheral ring *C* and dense inner ring *A* have expanded more or less symmetrically. *C* still has the characteristic triplex structure. Between *A* and *C* an incomplete triple layered ring has formed at *D* (two-thirds natural size)

Figure 3 shows another bull's eye colony of the same organism. The peripheral ring of this colony is triple-layered as usual, but the ring has been held back at one point by some unknown external obstruction or intrinsic weakness. Consequently, the two arcs on either side of the obstruction have bulged forward. But the symmetrical form of the resulting periphery seems to indicate a flexibility of the peripheral ring as a whole. This will be referred to in the discussion. The dense ring near the middle is not round but it is curvilinear rather than angular. It also shows a beaded or broken appearance called, for convenience, "bacterial swirl."

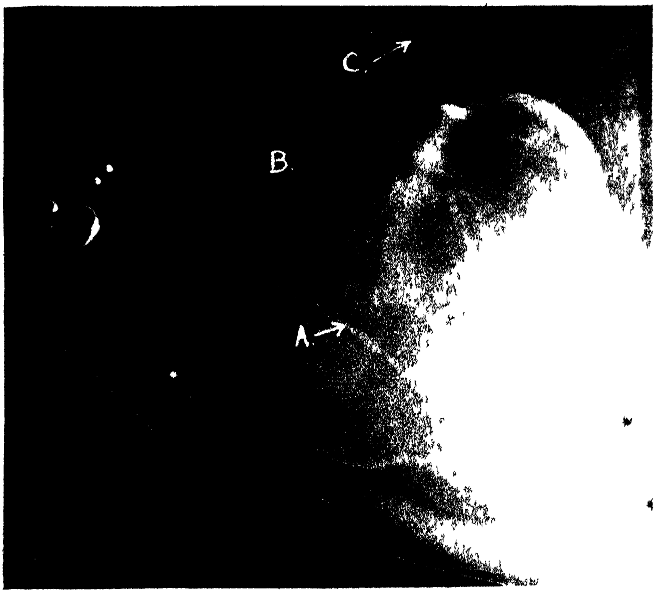

FIG 3 AN ASYMMETRICAL RING COLONY

Age, several hours. The dense inner ring, *A*, shows beady swirl. The peripheral ring, *C* has been held back at one place, and has bulged forward on either side to form a symmetrical curve. Same strain as figures 1 and 2 (two-thirds natural size).

This peculiar appearance apparently illustrates the communal action of the motile bacilli when they are in a formation and meet an external obstruction, or suffer some internal interference with their progression. If an area of the plate occupied by a peripheral ring is stirred with a sterile wire or other similar object, a uniform, homogeneous density results which, after a few minutes, changes into the beaded appearance characteristic of "swirl" formation. It is possible that lateral expansion causes a buckling and folding of the peripheral ring or other ring formation and thus leads to the beaded swirl. The motile organisms studied grew and progressed at a uniform rate of speed unless their progress was impeded by obstacles, in which event the appearance of the colonies changed.

In order to secure more evidence on the question of constancy and uniformity of progression of mobile colonies of bacteria, the following experiments were performed:

A dozen long tubes of semi-fluid media were set up. Three were inoculated with each of the following organisms: *Eberthella typhi; Salmonella schottmuelleri; Salmonella paratyphi;* and *Escherichia coli.* The progression of each was recorded at thirty-minute intervals for five such periods. There was no detectible variation in the half-hourly progression of any one of the twelve cultures. Twenty hours later the distance traversed was in the same proportion, amounting to about 4 inches in the case of *Eberthella typhi.* The three remaining organisms grew twice as fast. On another day, one long tube inoculated with *Salmonella schottmuelleri* was marked with ink at half-hourly intervals for twelve such intervals. Progression was uniform and constant.

The following previously unreported observations afford additional proof that bacteria progress constantly and at a uniform rate through liquid media.

Thirty-four long tubes containing liquid media were inoculated with an unidentified organism obtained from a toilet bowl on board an Army Transport. These were grown at room temperature during quiet weather in July, 1924, between Panama and San Francisco.

One hundred and six drawings were made of mobile colonies in these tubes. In some instances the outlines of the colonies were re-drawn at short intervals, varying from thirty seconds to three minutes. Invariably, change in shape was noticeable. Progression was always detectible within a few minutes. In some instances the bacteria which formed a layer in the tube were seen to heave themselves up in small elevations resembling wrinkles in a carpet. Within three minutes, the separate wrinkles would become detached and stand up higher. They would then change their shape constantly as they progressed through the tube. The motion and the vibration of the ship did not break up the mobile colonies, which in all essential respects resembled those seen in dozens of tubes inoculated with known strains and incubated on land.

DISCUSSION

The illustrations show that the bacteria studied rarely diffuse uniformly through liquid media. They usually form an orderly arrangement and proceed in a stereotyped and characteristic fashion. While some bacteria undoubtedly drop out of the formation from moment to moment, the bulk of the germ population proceeds in formation, leaving relatively clear medium behind. Later the bacteria left behind usually multiply and cause turbidity.

In some instances the bacteria fail to form mobile colonies. I have gotten the colonies best with heavy inoculum derived from an agar slant culture twenty-four hours old or much older.

Surface tension has been mentioned as a possible explanation of these expanding ring colonies. When the suspension of bacteria is first introduced into the thin horizontal plate, the characteristic triple-layered appearance of the periphery is not seen. This peculiar formation develops in cultures of motile, but not in those of non-motile, organisms. Its time of development corresponds with the time the organisms come out of lag. If the formation is disturbed with a wire, it changes into the beaded formation called "swirl," which is apparently a conglomeration of partially successful peripheral ring formations. For these reasons, it appears that the peripheral ring represents a primitive communal activity of bacteria, remotely resembling the parallelism of blackbirds flying in a flock.

With reference to the ring colony shown in figure 1, if any segment of the circle slowed down, it would ruin the alignment and symmetry of the colony. The circular form of the colony therefore proves that either there was continuous progression, or that there was an organization enabling the different parts of the colony to proceed in unison. This latter is absurd. Continuous progression is the only satisfactory explanation.

In this connection Rogers and Greenbank (1930) have used the long tube method for cultivating *Escherichia coli* and have reported intermittency of growth. They, however, wound their culture tube in spiral form. This rendered a profile view of the mobile colonies impossible, and they depended upon photographs of indicator color to estimate progress. This apparently leads to undesirable vagueness. These observers report it exceptional for the organism cultured to maintain a uniform progression for two hours successively. The spiral tube as used by Rogers and Greenbank apparently does not afford the profile view necessary for observing mobile colonies. In early experiments the present author had not observed mobile colonies. There was intermittency of progression in the curved tube previously illustrated. After mobile colonies were first seen, and upon repetition of similar experiments, uniform progression was found to be the rule, even with the S3 strain, which had earlier apparently grown intermittently. Some of my earlier experiments therefore gave results in accord with those of Rogers and Greenbank. No organism was however cultured which did not under proper conditions progress smoothly and uniformly.

The author's observation of many hundreds of mobile colonies in long tubes and several dozens in horizontal liquid plates have shown conclusively that all motile strains tried could be made to progress uniformly and constantly.

Moreover, there is a distinct difference in the gross appearance of bacteria in lag and those actively progressing. One who has become accustomed to the appearance of mobile colonies can recognize instantly when they are formed up and on the move.

For opportunity to carry on these experiments, the author is indebted to Colonel Thomas R. Bratton, Medical Corps, United States Army, and Major Albert G. Kinberger, Medical Corps, United States Army.

SUMMARY AND CONCLUSIONS

1. Motile bacteria have been grown and photographed in thin sheets of liquid media held by capillarity between panes of glass.
2. Some of the motile organisms formed two or more peculiar

concentric rings which expanded as the colony enlarged to occupy the available space.

3. The speed of growth and progression of the motile organisms was constant and uniform.

4. If the formation of actively motile organisms was broken up by mechanical means, the bacteria soon took on a peculiar appearance called bacterial "swirl."

5. The phenomena observed suggest that the bacteria have a form of sensitiveness causing them to respond to the proximity of other similar organisms, particularly those of their own species.

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THE IDENTITY OF BACILLUS PUTRIFICUS BIENSTOCK

ANDREW CUNNINGHAM

Department of Bacteriology, College of Agriculture, Edinburgh

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In the literature on the putrefactive, anaerobic bacilli there is, as Reddish and Rettger (1922) have pointed out, much confusion regarding the identity of *Bacillus putrificus* Bienstock. This is largely due to the fact that Bienstock's first publication (1884) refers to cultures which were impure, as he himself later admitted, and that the subsequent papers of this author (1899, 1901, 1906) contain imperfect descriptions of his organism which are to some extent mutually contradictory.

The majority of investigators are agreed that Bienstock's bacillus is an anaerobic, motile, sporulating rod which liquefies gelatin, peptonises milk and is non-pathogenic. In the published descriptions of this bacillus there is some diversity of opinion as to the rate at which the organism is capable of liquefying gelatin and peptonising milk. The main characteristics about which there is disagreement, however, are the shape and position of the spores, the size of the rods and the ability of the organism to ferment carbohydrates. Certain workers describe *Bac. putrificus* as a slender rod, which produces more or less spherical spores, always strictly terminal and which is incapable of attacking sugars to any appreciable extent. The chief advocates of this view are Reddish and Rettger (1922, 1923, 1924). On the other hand several investigators have referred to the fermentation of certain carbohydrates by strains of *Bac. putrificus* obtained from type culture collections (Achalme (1902); Reddish (1924)) or by cultures believed to be identical with Bienstock's bacillus (Kendall, Day and Walker (1922); Kahn (1922)).

In the course of an investigation of certain anaerobic bacilli a number of strains of sporulating anaerobes which resemble more or less closely the forms referred to in the literature as *Bac. putrificus* have been examined. The organisms studied have already been described in detail in a previous paper (1931) under the numbers B_{4a}, B_{4b}, and B_5. B_5 (fig. 3) is the form which Reddish and Rettger consider to be identical with Bienstock's bacillus; B_{4b} (fig. 4), often referred to as *Bac. sporogenes* Metchnikoff, is the gas-producing type which certain workers regard as *Bac. putrificus*, (see Medical Research Committee, 1919, p. 94). In some of its characteristics B_{4a} (figs. 1 and 2) is intermediate between B_5 and B_{4b} and it corresponds more closely to Bienstock's description than either of these forms.

The studies on which the descriptions referred to (1931) are based were made on six strains of B_{4a}, four strains of B_{4b} and five strains of B_5, isolated during the investigation, and on the following type cultures:

(1) *Bac. putrificus verrucosus* Stamm 22.	Received from Prof. Zeissler, Altona-Elbe.
(2) *Bac. putrificus verrucosus* Stamm 58.	
(3) *Clostridium putrificum* (Bienstock) Bergey et al. No. 3559 (Hall collection No. 38)	Obtained from the American Type Culture Collection.
(4) *Clostridium putrificum* (Bienstock) Bergey et al. No. 679	
(5) *Clostridium putrificum* Sturges No. 2221	Received from the National Collection of Type Cultures, London.
(6) *Bac. cochlearius* Douglas, Fleming and Colebrook. T. M. I. H. C. No. 535	

Nos. 1 and 2 were found to be identical with B_{4b}; Nos. 3 to 6 with B_5.

Reddish and Rettger (1923) reject Bienstock's 1884, 1899 and 1901 descriptions as worthless on the ground that his cultures were impure. Nevertheless they state that these descriptions certainly "had reference to an anaerobe having round terminal spores." If the cultures on which the descriptions are based were impure, it is impossible to assert that the spores observed belonged to *Bac. putrificus* and not to a contaminant. Reddish and Rettger, however, accept the 1906 description, regarding which Reddish (1924) states that it furnishes "the final criteria for our guidance in this work." Bienstock's admission that in 1884 he worked with impure cultures, has already been noted. If his 1906 characterisation is accepted as accurate the objection raised by Reddish and Rettger to the 1899 paper also appears to be justified, because the 1899 and 1906 descriptions are not in agreement. In Bienstock's 1899 paper, although growth in ordinary agar is referred to, no mention is made of gas formation in this medium, yet the organism is described as frequently producing gas in glucose agar. In the 1906 publication, however, the fact that *Bac. putrificus* is incapable of fermenting glucose is emphasised. These two statements appear to be irreconcilable. The 1901 paper contains little of diagnostic value.

A careful study of the 1906 paper indicates that there are several respects in which Reddish and Rettger's organism does not correspond with Bienstock's description. The latter author states that in certain media ("l'albumine cuite") his bacillus frequently produces, in addition to drumstick shaped rods, the clostridium type of sporulation (that is, central spores). B_5 (Reddish and Rettger's bacillus) has not been observed to produce clostridia and no reliable data on this type of sporulation in cultures of the organism have been encountered in the literature. On the other hand B_{4a} may produce a small proportion of clostridia when grown on beef-infusion agar (fig. 2). The majority of the spores of this organism are, however, formed very close to the ends of the rods and many are definitely terminal (figs. 1 and 2). The proportion of terminal to subterminal spores varies in different strains and even in the same strain grown on a variety of media, and in certain cases terminal spores may predominate.

Reddish and Rettger (1922) claim that Bienstock's expression "baguette de tambour" should be interpreted as meaning that the spores of *Bac. putrificus* are terminal and spherical. They appear to base their opinion on the fact that in its sporulating stage *Clostridium tetani* is described quite generally in the literature as a drumstick form. It is clear, however, that this usage of the term does not necessarily restrict its application to rods with spherical spores. At an earlier stage the authors themselves admit that some investigators have interpreted the phrase as implying that the spore is ovoid. Sporangia containing either spherical or ovoid spores in a terminal position may be equally correctly referred to as "baguettes de tambour." Reddish and Rettger describe the spores of their organism as round and wider than the cells. When they are first formed they may be spherical but ultimately they become distinctly ovoid (fig. 3). The spores of B_{4a} are ovoid and in certain cases almost cylindrical; they are generally wider than the sporangia (figs. 1 and 2).

The action of Reddish and Rettger's organism on milk is not in accordance with the 1906 description of *Bac. putrificus*. Bienstock states that his bacillus produces putrefactive changes in milk. These begin after about twenty-four hours and are rapidly completed. The medium is peptonised, amino- and other organic acids are formed and a putrefactive odour develops. Observations made in the course of this investigation, as well as by Hall (1922), indicate that Reddish and Rettger's bacillus is incapable of producing rapid peptonisation of milk when examined by the methods generally in use. Hall examined two Sturges strains, isolated in Rettger's laboratory, and found that they had no action on milk. The majority of the strains of B_5 when first isolated also produced no change in milk. Certain strains, however, when retested showed a slow peptonisation without curdling after one to two months' incubation at 37°C. In all, twenty-four tubes of milk were inoculated from pure cultures of the organisms. Of these, twenty remained unchanged in appearance at 37°C. for three weeks or longer; in the remainder a slow peptonisation took place. Reddish and Rettger (1923) have observed that in tubes of milk, each inoculated with a large loopful of a six weeks old egg-meat culture of their bacillus, slight digestion occurred at the end of twenty-four hours and practically complete digestion within four days. The rapidity of the action is attributed to the presence of preformed enzymes carried over in the inoculum. Such mass inoculations are, however, unusual in bacteriological practice. In a later paper Reddish (1924) describes his milk cultures as showing digestion only after two weeks' incubation. Type B_{4a} is much more active than B_5. Milk inoculated in the usual way from young cultures on glucose or ordinary agar shows, after two to three days' incubation at 37°C., a soft curd with separation of a small quantity of clear liquid at the surface. After approximately twenty-four hours' incubation curdling has been observed in milk inoculated from old bullock's heart cultures and occasionally in that inoculated from young cultures on beef-infusion agar. Peptonisation becomes obvious almost immediately after the curd has been formed and proceeds more or less rapidly. The liquid becomes transparent in parts but is never completely cleared. It appears whitish and more or less turbid and has a cheesey odour. This stage is reached in from ten days to about one month, after which no further change takes place.

Bienstock states that the putrefactive activity of his bacillus is retarded or inhibited by the presence of *Bac. coli* or *Bac. lactis aerogenes.* In order to study the effect of the latter organisms on the putrefactive changes produced by B_{4a} a number of tubes of milk were inoculated with this bacillus. Half of the tubes then received a further inoculation of *Bac. coli* or *Bac. aerogenes* and all were incubated anaerobically at 37°C. In all the cultures prompt precipitation of the casein took place. After a total incubation period of about three weeks the appearance of the curd in those tubes which had received inoculations of *Bac. coli* or *Bac. aerogenes* remained practically as it was when curdling first took place, whereas in the tubes containing B_{4a} alone the characteristic peptonisation change was complete. It appears, therefore, that *Bac. coli* and *Bac. aerogenes* are capable of inhibiting the peptonisation of milk by B_{4a}.

Owing to the variation in the rate of liquefaction of gelatin by different strains of the same type as well as by the same strain at different times, it is difficult to give definite comparative figures for B_{4a} and B_5. In general, however, it may be stated that the former liquefies gelatin more rapidly than the latter. In the case of B_{4a} liquefaction of gelatin frequently takes place after three to four days' incubation at 22°C. whereas in that of B_5 it has not been observed to occur in less than about ten days. Gelatin, inoculated from one of the B_5 strains and incubated at 37°C., solidified on cooling even after an incubation period of more than three months. It may be recalled that *Bac. cochlearius,* since identified with Reddish and Rettger's organism (Hall (1922); Fildes (1929); Cunningham (1931)) was originally described as incapable of liquefying gelatin. The latter observation was attritubed by Hall to the difficulty in obtaining growth of the organism in the medium. Strain No. 535 from the National Collection of Type Cultures, London, examined during this investigation also showed some reluctance to grow in gelatin at 37°C. Absence of liquefaction of gelatin by the strain of B_5 referred to above was not, however, due to its failure to grow in the medium.

In motility, Gram-staining reaction and the characteristics of their growth in bouillon, bullock's heart medium, brain and liver-liver bouillon types B_{4a} and B_5 show marked similarity. B_5, however, grows much more slowly than B_{4a} and, therefore, it is improbable that organisms of the former type could have produced the changes in certain media described by Bienstock in the comparatively short time during which it was usual to keep cultures under observation at that period.

Strains of B_5 are difficult to isolate in pure culture. Thus, Sturges and Rettger (1919) and Würcker (1909), whose *Bac. postumus* was probably also of this type (Zeissler (1928)), experienced difficulty in obtaining colonies of the organism on solid media and had to devise special methods for its isolation. Würcker states that he failed to obtain growth of his bacillus on agar (from the context it appears that glucose agar was used, p. 230) in plate and shake cultures. When, however, he employed as the basis of his agar medium horse liver, previously digested for fourteen days by a putrefactive anaerobic bacillus, he secured colonies and succeeded in isolating the organism. Sturges and Rettger observed that *Clostridium putrificum* only developed on ordinary agar plates incubated in a Novy jar when it was associated with other organisms. They obtained the bacillus in pure culture by growing it with *Bac. coli* or *Staphylococcus aureus* until spores were produced in the mixed colonies so formed and heating the cultures to destroy the non-sporing organisms. Hall (1922) emphasises the tedious nature of the work involved in obtaining colonies of this bacillus on solid media in order to verify the purity of cultures. B_{4a}, however, can be readily isolated by anaerobic plating on ordinary agar.

Bienstock does not describe his isolation methods in the 1906 paper. In a previous publication (1899, p. 351) he gives an account of the isolation of his bacillus, from which it appears that he experienced no particular difficulty in obtaining colonies in deep tubes of glucose agar or gelatin. The negative results obtained by Würcker when he used glucose agar shake cultures, the difficulty experienced by Sturges and Rettger in obtaining colonies of their organism and the tedious nature of the technique involved in the isolation of B_5 even when modern methods are employed, all raise doubt as to whether Bienstock's methods were capable of producing growths of an organism of this type on the solid media employed by him.

Bienstock's 1906 description of *Bac. putrificus* is also at variance with the opinion of those workers who believe that this organism is capable of fermenting certain sugars. Bienstock is most emphatic in declaring that his bacillus was incapable of attacking carbohydrates. When first isolated, the strains of B_{4a} showed no action upon the common sugars and other substances generally employed in fermentation tests. The same strains, when retested after they had been in pure culture for some time, produced slight acidity and minute amounts of gas in peptone water containing certain sugars including glucose. When, however, they were grown in glucose agar stabs, they consistently failed to produce gas. It may, therefore, be concluded that they show no appreciable fermentative activity towards the usual test substances.

The facts already recorded indicate clearly that the characteristics of B_{4a} correspond more closely to those of Bienstock's organism, as described in his 1906 paper, than do those of *Clostridium putrificum* of Reddish and Rettger or the gas-producing bacillus considered by certain other workers to be identical with *Bac. putrificus*. If Bienstock's incomplete description can be accepted at all as an adequate characterisation of his organism, B_{4a} is, of all the anaerobic bacilli which have hitherto been accurately and fully described, the one which agrees most closely with this characterisation. It is suggested, therefore, that the name *Bac. putrificus* Bienstock should be reserved for this organism.

The literature on the anaerobic bacilli contains very few descriptions of organisms of this type. The bacillus referred to by Hempl (1918) as organism I, which was isolated from septic wounds and completely described, appears to be identical with B_{4a}. *Bac. sporogenes-regularis* of Distaso (1911) and *Bac. sporogenes-parvus* of Choukevitch (1913) are probably also closely allied to, or identical with, this organism but the meagre descriptions of the authors referred to do not permit identification to be made with certainty.

The adoption of the name *Bac. putrificus* for organisms of the B_{4a} type will necessitate the renaming of *Clostridium putrificum.* In 1919 the Medical Research Committee described an anaerobic bacillus which they named *Bac. cochlearius* Douglas, Fleming and Colebrook. As already indicated, original strains of this organism have been identified with *Clostridium putrificum* by a number of workers (Hall (1922); Fildes (1929); Cunningham (1931)). There appears, therefore, to be justification for using the name *Bac. cochlearius* for organisms of this type. The Medical Research Committee's description, however, requires to be modified in at least two particulars. It contains the statements that the organism does not liquefy gelatin and that it has no action on milk. The majority of strains (including original strains of *Bac. cochlearius*) however, liquefy gelatin slowly, and some are also capable of producing slow peptonisation of milk. The Medical Research Committee's description should, therefore, be modified to include strains which produce slow liquefaction and peptonisation. In general, organisms which produce slow liquefaction of gelatin and peptonisation of milk have more in common with those which are without action on these media than with cultures which bring about rapid liquefaction and peptonisation. They should, therefore, be classed with the former.

I am indebted to Messrs. T. Hamilton and W. Watson of the Royal College of Physicians Laboratory, Edinburgh, for the microphotographs which accompany this paper.

SUMMARY

An anaerobic sporing bacillus is described, the characteristics of which correspond more closely with Bienstock's final description of *Bac. putrificus* than those of any other organism hitherto described. It is proposed that the name *Bac. putrificus* Bienstock should be reserved for this organism.

The adoption of this name for the organism in question will involve the renaming of *Clostridium putrificum* Reddish and Rettger. It is suggested that the name *Bac. cochlearius* Douglas, Fleming and Colebrook should be used when reference is made to this bacillus.

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PLATE 1

Figs. 1 and 2. *Bacillus putrificus.* Beef-infusion agar. Two days at 37°C. Rods and endospores. × 1000.

Fig. 3. *Clostridium putrificum.* Beef-infusion agar. Nine days at 37°C. Rods and endospores. × 1000.

Fig. 4. *Bacillus sporogenes.* Ordinary agar. Two days at 37°C. Rods and endospores. × 1100.

FIG. 1

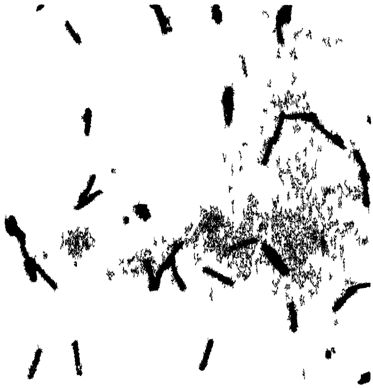

FIG. 2

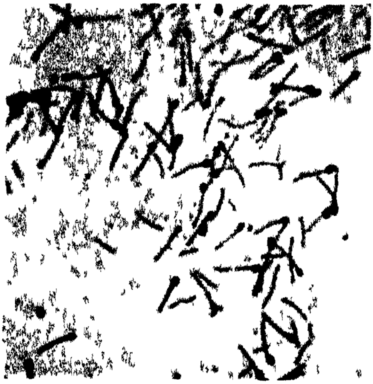

FIG. 3

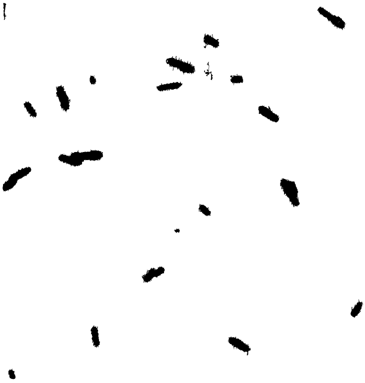

FIG. 4

(Andrew Cunningham Identity of *Bacillus putrificus* Bienstock)

STUDIES ON LACTOBACILLUS ACIDOPHILUS AND LACTOBACILLUS BULGARICUS

II. ELECTROPHORESIS STUDIES

R. H. WEAVER

Associate Professor of Bacteriology, University of Kentucky, Lexington, Kentucky

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That the electrical charge (P.D. or electrical-potential difference between a particle and its menstruum as postulated in the Helmholtz double-layer theory) carried by a bacterium, indicates or determines some of its physiological characteristics is evident from the studies of various investigators. Bechhold (1904) seems to have been the first to report that bacterial cells carry a negative charge. Since that time this observation has been confirmed many times although a few workers, e.g. Cernovodeanu and Henri (1906), have claimed to have found a few individual species which carried the opposite charge. An excellent review of this early work may be found in a paper by Winslow, Falk, and Caulfield (1923). The electrical charge may be reduced or even reversed in the presence of acids or alkalies, or in the presence of electrolytes, especially the salts of trivalent ions such as lanthanum. A few substances such as sodium oleate may increase the charge.

In a study of the electrical charges of organisms and their surface tension (apparently the interfacial tension between the organism and the medium) Girard and Audubert (1918) found that lowering the charge—increasing the surface tension—by the use of lanthanum nitrate, increased the growth of the organism, increased the longevity of the organism in the medium, and caused it to maintain its toxicity. Shearer (1922) was able partially to confirm Girard and Audubert's findings. Winslow, Falk, and Caulfield stated that "marked deviation from ordinary potential difference is very probably incompatible with viability. . . . . We have pointed out that certain potential differences between bacteria and their menstrua are apparently associated with some of the phenomena of viability." Eggerth (1923) (1924) was of the opinion that changes in electrical charge ran more or less parallel to changes in viability, at least in buffered solutions. In contrast to this was the work of Northrop and DeKruif (1922) and of Winslow and Fleeson (1926) in which the effects produced by altering the electrical charge seemed to be only remotely related to vital phenomena.

Falk and his associates (Falk, Gussin and Jacobson (1925); Falk, Jacobson, and Gussin (1925); Falk and Jacobson (1925) (1926)) in a study of the virulence of pneumococci reported that there were significant parallelisms, direct or inverse, between electrophoretic potential, virulence for white mice and fatality for man, agglutinability, colony formation, phagocytic reactions or opsonic indices, sodium oleate or bile solubility and other characteristics of these bacteria. Later, Falk and his associates (Jensen and Falk (1928); Jensen, Falk, Tonney, and White (1928); Falk, Jensen, and Mills (1928)) showed that there was an inverse relationship between the electrophoretic potential and the toxicity of the diphtheria bacillus, and that when the electrophoretic potential was altered the toxicity was correspondingly altered.

Winslow and Upton (1926), working with different types of vegetable cells, found that the electrical charges carried by various species were different and that the addition of electrolytes did not have the same degree of effect upon the charge of each type. In a study of the electrophoretic potential as an aid in the identification of strains of the *B. coli* group, Chapman (1929) found that individual strains carried a characteristic charge, which was, however, constant for each dissociant. He was able to standardize his technique so that a constant figure could be obtained for the potential of any strain.

In view of these observations that species of bacteria and even individual strains of the same species carry characteristic electrophoretic charges studies were undertaken with a group of strains of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*, in the hope that the findings might indicate something of the difference in physiological nature which exists between the two groups of organisms and might lead to a reliable means of selecting implantable strains (*Lactobacillus acidophilus*). Such a procedure seemed especially likely to produce valuable results, since the reports by Albus and Holm (1926) and by Kopeloff and Beerman (1927) that a difference exists in the ability of the two species to resist a lowered surface tension of the medium. Attention has already been called to the relationship which seems to exist between the surface tension of the organism and the electrical charge carried by it.

STRAINS USED

For the strains used in this study the writer is indebted to Dr. M. Scherago, Dr. W. L. Kulp, Dr. A. A. Day, Dr. N. Kopeloff, and the American Type Culture Collection. Most of these cultures have been used in extensive investigations of the lactobacilli by various workers, many have been employed in implantation experiments, and on the whole they constitute as authentic a group of cultures as could be obtained. Many of these cultures were apparently duplicates but as this seemed to add something to the investigation no attempt was made to eliminate them. A brief summary of the source and history of the 20 strains of *L. acidophilus* and of the 11 strains of *L. bulgaricus* used follows.

L. acidophilus (*according to donor*)

AK 1129—Scherago—isolated from human feces January 12, 1929. Implantable.
FBC 5209—Scherago—isolated from human feces May 20, 1929. Implantable.
YE 2169—Scherago—isolated from human feces February 16, 1929. Y strain.
YR 2209—Scherago—isolated from human feces February 20, 1929. Y strain.
LAK 328—Scherago—isolated from human feces March 2, 1928.
KOP—Kopeloff—reisolated from treated patient 1929.
R-1-1 KO—Kopeloff—apparent duplicate of R-1-1 KU.
Scav—Kopeloff—apparent duplicate of 833.

Torrey—Kopeloff—probably Torrey's strain "m" isolated from human feces.

AL 34 KO—Kopeloff—apparent duplicate of AL 34 KU, called *L. bulgaricus* by Kulp.

R-1-1 KU—Kulp—isolated from rat 1924. Implantable.

4B—Kulp—isolated from human 1926. Implantable.

833—Kulp—American Type Culture Collection strain.

Ha X—Kulp—isolated from human 1927. Implantable.

Acid II Bass—Day—From Bass 1923.

Acid II RCH—Day—isolated 1924 from feces of human treated with Acid II Bass.

Acid II PI—Day—isolated 1924 from feces of human treated with Acid II Bass.

Acid R—Day—isolated from adult human feces.

Lederle—Day—isolated 1924 from Lederle acidophilus milk.

Acid III—Day—obtained prior to 1924 from Service Laboratory of New York.

L. bulgaricus (according to donor)

Day—Day—isolated from milk by Kendall, 1923.

B 4 KU—Kulp—obtained from Sherman. Not implantable.

AL 34 Ku—Kulp—obtained from Sherman. Maltose positive.

B 12 KU—Kulp—obtained from Sherman. Not implantable.

B 12 KO—Kopeloff—apparent duplicate of B 12 KO.

B 4 KO—Kopeloff—apparent duplicate of B 4 KU. Not implantable.

B 2 US—Kopeloff—obtained from Albus.

B 3—Kopeloff—obtained from Kulp. Not implantable.

Kingman—Kopeloff—obtained from Kingman. Reported originally from Metchnikoff.

521—American Type Culture Collection—originally from Metchnikoff.

521 N—American Type Culture Collection—duplicate of 521. Obtained from collection two years after 521.

PROCEDURE

For the determination of the electrical charge (P.D.) of the organism the Falk cell (slide type) as described by Falk, Jensen, and Mills was used. This type of apparatus was found to give satisfactory results, provided the cement which covered the platinum wires was exactly flush with the surface. Otherwise air was sucked into the chamber. Some of the new cells were not satisfactory in this respect and had to be recemented. All of the cells had to be recemented occasionally when they were in constant use.

Stock cultures were maintained in milk. Pipette transfers were made from these milk cultures into tomato juice broth, (Kulp (1927)), which was adjusted so as to give a hydrogen ion concentration of pH 6.8 to 7.0 after sterilization. After two generations of growth in this medium, to acclimatize the organisms and to get rid of the milk carried over by the first transfer, suspensions were made by centrifugalizing, washing three times, and suspending in distilled water. Duplicate cultures of each strain were examined. The technique of the measurement of the electrical charge was identical with that described by Falk, Jensen, and Mills.

RESULTS

The results from a preliminary study of the electrical charges carried by four cultures of *L. acidophilus* and one of *L. bulgaricus* are shown in table 1.

Organisms from culture FBC 5209, which had been isolated only two months previously showed extremely low electrophoretic mobilities. Individual organisms showed considerable variability in their P.D., some apparently carrying no charge at all, and a few carrying a weak positive charge. The recorded mobility of 7.8μ per second was obtained by disregarding those organisms carrying a positive charge and is, therefore, too great. Strain AK 1129, an X strain, which had been isolated six months previously showed a mobility of 21.6μ per second. Two Y strains, YR and YE, showed mobilities of 27.0 and 30.6 respectively and the one strain of *L. bulgaricus* a mobility of 32.9.

In view of the appreciable difference shown in the electrophoretic mobilities of organisms from the X strains of *L. acidophilus* and those from the one strain of *L. bulgaricus*, the investigation was continued using 26 additional cultures, which were obtained from reliable sources. Repeated measurements of the electrophoretic mobilities of these cultures were made over a period of six months. The results of one typical experiment are included in table 2.

Thirteen of the 20 cultures labelled *L. acidophilus* by their donors showed lower electrophoretic mobilities (between 11.3μ and 26.0μ per second), than any of the cultures labelled *L. bulgaricus*: 2 showed mobilities of 26.6μ per second, and 5 gave results that were considerably higher. Of the 5 which showed mobilities comparable to those of the average *L. bulgaricus* strains, at least 4 may be considered as doubtful with regard to their classification. According to available records R–1–1 KO and Scav were duplicates of R–1–1 KU and 833 respectively, but they were not similar to them in morphology or in cultural growth. Apparently, in several years of culture in the hands of different individuals, either considerable variation had taken place in these cultures, or else the identity of the strains had become confused. YR 2209 was a consistent Y strain and could not be classed as *L. acidophilus* according to the standards of some authorities. AL 34 KO was shown by the records and by its peculiar colony form to be identical with AL 34 KU. AL 34 KU was labelled as an atypical strain of *L. bulgaricus* by its donor and AL 34 KO as *L. acidophilus* by its donor. The average electrophoretic mobility of the 20 cultures labelled *L. acidophilus* was 23.7μ per second and that of the 11 *L. bulgaricus* cultures 31.5μ per second.

TABLE 1

Electrophoretic mobilities of distilled water suspensions from three-day tomato juice broth cultures

CULTURE	NAME ACCORDING TO DONOR	P.D.
		μ/second
FBC 5209	*L. acidophilus*	<7.8
AK 1129	*L. acidophilus*	21 6
YR 2209	*L. acidophilus*	27.0
YE 2169	*L. acidophilus*	30.6
521	*L. bulgaricus*	32 9

The data from repeated experiments, similar to the one just described are too voluminous to be recorded here. With one exception they agree almost exactly with the one reported. About one month after the beginning of this series of experiments strain AK 1129 underwent what was apparently a sudden variation. While it, previously, had coagulated milk in twenty-four hours, it now required about five days. Previously it had ranked among

TABLE 2

Electrophoretic mobilities of distilled water suspensions from three-day tomato juice broth cultures

CULTURE	NAME ACCORDING TO DONOR	P.D. μ/second
AK 1129	*L. acidophilus*	11 3
KOP	*L. acidophilus*	15 2
R-1-1 KU	*L. acidophilus*	18 1
4B	*L. acidophilus*	18.9
FBC 5209	*L. acidophilus*	18 9
833	*L. acidophilus*	20 4
Acid II Bass	*L. acidophilus*	20 7
LAK	*L. acidophilus*	20 8
HA X	*L. acidophilus*	21 2
Acid II RCH	*L. acidophilus*	22.6
YE 2169	*L. acidophilus*	25 0
Acid II PI	*L. acidophilus*	25 8
Lederle	*L. acidophilus*	26 0
Day	*L. bulgaricus*	26 0
Acid R	*L. acidophilus*	26 6
Acid III	*L. acidophilus*	26 6
B4 KU	*L. bulgaricus*	26.9
R-1-1 KO	*L. acidophilus*	27.5
YR 2209	*L. acidophilus*	29 2
AL 34 KU	*L. bulgaricus*	29.6
B 12 KU	*L. bulgaricus*	29.8
521	*L. bulgaricus*	31 1
B 12 KO	*L. bulgaricus*	31.4
Torrey	*L. acidophilus*	32.1
AL 34 KO	*L. acidophilus*	32.2
521 N	*L. bulgaricus*	33.4
Kingman	*L. bulgaricus*	33 4
B 2 US	*L. bulgaricus*	33 7
B 3	*L. bulgaricus*	34 1
Scav	*L. acidophilus*	35.0
B 4 KO	*L. bulgaricus*	37.5

the higher *L. acidophilus* strains with respect to its electrophoretic mobility, now it showed the lowest mobility of all. The variation was apparently permanent because this strain retained its new characteristics throughout the remainder of the experiments. Due to various factors which were not controlled the electrophoretic mobilities shown by any culture on different days were not the

TABLE 3

Sequence of cultures according to average rank determined from 4 electrophoretic mobility measurements (November 30, December 23, March 10, and March 24)

	CULTURE	AVERAGE RANK	NAME ACCORDING TO DONOR	IMPLANTATION RESULTS
1	4B	5.0	*L. acidophilus*	+ (Kulp)
2	FBC 5209	5.5	*L. acidophilus*	+ (Scherago)
3	R-1-1 KU	5.5	*L. acidophilus*	+ (Kulp)
4	AK 1129	6.0	*L. acidophilus*	+ (Scherago)
5	HA X	7.25	*L. acidophilus*	+ (Kulp)
6	KOP	7.25	*L. acidophilus*	+ (Kopeloff)
7	833	7.5	*L. acidophilus*	No record
8	Acid II Bass	9.0	*L. acidophilus*	+ (Bass) (Day)
9	Lederle	9.75	*L. acidophilus*	No record
10	LAK 328	9.75	*L. acidophilus*	No record
11	Acid II RCH	11.25	*L. acidophilus*	No record
12	YE 2169	11.5	*L. acidophilus*	No record
13	Acid II PI	13.0	*L. acidophilus*	No record
14	Acid III	15.0	*L. acidophilus*	No record
15	Acid R	15.5	*L. acidophilus*	No record
16	B4 KU	16.0	*L. bulgaricus*	No record
17	Day	16.25	*L. bulgaricus*	No record
18	Torrey	17.5	*L. acidophilus*	No record
19	YR 2209	18.0	*L. acidophilus*	No record
20	AL 34 KO	22.25	*L. acidophilus*	No record
21	R-1-1 KO	22.25	*L. acidophilus*	No record
22	521 N	23.0	*L. bulgaricus*	No record
23	AL 34 KU	23.0	*L. bulgaricus*	No record
24	B 12 KO	23.25	*L. bulgaricus*	No record
25	521	24.25	*L. bulgaricus*	No record
26	B 12 KU	25.5	*L. bulgaricus*	− (Kulp)
27	B 2 US	25.75	*L. bulgaricus*	No record
28	B 4 KO	26.5	*L. bulgaricus*	− (Kopeloff)
29	Kingman	27.25	*L. bulgaricus*	No record
30	B 3	27.75	*L. bulgaricus*	− (Kopeloff)
31	Scav	30.25	*L. acidophilus*	No record

same; however, the position of that culture in the series arranged according to the mobilities was approximately the same. A summary of the results of 4 experiments in which all 31 strains were used is found in table 3. For reasons mentioned above it seemed to be fairer to summarize the results by arranging the strains in a series for each experiment according to the mobilities and then to take an average of the position of the strains in the series rather than to take an average of the mobilities. This procedure was followed for table 3.

Fifteen of the 20 strains labelled *L. acidophilus*, according to their average rank, carried a lower electrical charge as shown by electrophoretic mobility, than any of the cultures labelled *L. bulgaricus*: 4 were placed above 2 of the 11 *L. bulgaricus* strains and the other one occupied the highest position in the series. As was previously stated there is good reason to doubt the correctness of identification of at least 4 of these latter 5 strains. Where definite records of positive or negative results with implantation experiments were available such information was included in the table. Since there appeared to be so much danger of mixing the identity of strains, any implantation experiments carried out by others than the direct donor of any strain were disregarded. It is of probable significance that records of positive implantation experiments are available for 7 of the lowest 8 members of the series and that records of negative results are available for 3 of the 6 highest members of the series.

DISCUSSION

An investigation of the electrical charges carried by the organisms of 31 "authentic" strains of *L. acidophilus* and *L. bulgaricus* by means of measuring their electrophoretic mobilities in a Falk cell (slide type) has led to the following observations.

1. The various strains did not fall into two distinct groups but arranged themselves in a series. This is in agreement with the observations of Falk and his associates on the toxigenic and nontoxigenic strains of *C. diphtheriae*. It also helps to justify the statement of Kulp and Rettger that "because of the various close relationships, it is suggested that these two types be placed

in one and the same species of which *L. acidophilus* is the central type and *L. bulgaricus* a variant."

2. With the exception of a few strains whose identity may be questioned, all cultures which were labelled *L. acidophilus* by their donors carried lower electrical charges than those which were labelled *L. bulgaricus*.

3. Most of the more recently isolated strains of *L. acidophilus* carried lower electrical charges than the majority of the older strains.

4. Standard strains of *L. acidophilus* which had been shown repeatedly to be implantable carried the lowest electrical charges of all strains in the series and similar strains of *L. bulgaricus* carried the highest charges.

These observations would seem to indicate that there is some very definite relationship between implantability and the electrical charge carried by the organisms of any particular strain. No definite standard for the separation of *L. acidophilus* and *L. bulgaricus* strains or for the separation of implantable from non-implantable strains can be established without further investigation, but it is suggested that valuable presumptive evidence may be obtained by a comparison of the electrophoretic mobilities of unknown cultures with those of such standard strains as 4B, FBC 5209, or R-1-1.

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STUDIES ON BACTERIAL SPORES

III. A CONTRIBUTION TO THE PHYSIOLOGY OF SPORE PRODUCTION IN BACILLUS MYCOIDES

B. C. BRUNSTETTER AND C. A. MAGOON

Division of Horticultural Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture

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The first study of this series (Magoon (1926a)) reported the effect of age, temperature and humidity on the thermal resistance of spores of *B. mycoides.* These spores, washed free of metabolic products, were stored at 10°C., 20°C., and 30°C., under three conditions of humidity—over calcium oxide, over 50 per cent H_2SO_4, and over water. Samples from each of the nine sets of storage conditions were tested for thermal resistance (as measured by the minutes of heating at 100°C. required for 100 per cent destruction of spores) after one, thirty, sixty, ninety, one hundred and twenty, one hundred and fifty and one hundred and eighty days of storage, respectively. It was found that the thermal resistance of spores had increased during thirty days under all sets of storage conditions. Though changes after thirty days were rather irregular, it was outstanding that the slowest change in resistance took place at 10°C. over CaO. Considering the storage period as a whole, it was found that at 10°C. the thermal resistance of the spores increased regularly with the humidity. At 20°C. it was about the same over CaO and 50 per cent H_2SO_4 and was slightly greater over water, while at 30°C. it was maximum over 50 per cent H_2SO_4 and least over water. Under the three conditions of humidity the maximum thermal resistance was found at 20°C.

In the second report (Magoon (1926b)) experiments were described in which by cultivation and selection of survivors from successive thermal death-time tests, a strain of *B. mycoides* was

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obtained whose spores had attained a resistance at least 25 times that of the original spores.

These results emphasized the fact that the thermal resistance of spores within a given bacterial species is not a fixed property and showed that if the spore problem is to be solved satisfactorily more information must be obtained in regard to the factors responsible for the resistance of spores to heat and for variations in that resistance; in other words, a thorough understanding of the biology of spore formation is required. Such a biological understanding is highly desirable as a foundation for a biochemical study of the process of spore formation and of the nature of the resistance of spores to heat.

Microscopic observation shows that the production of spores by vegetative cells follows a definite sequence of changes in the internal structure of the cell. It is necessary, therefore, if a satisfactory chemical or physical analysis is to be made of the factors responsible for the greater thermal resistance of the spores as compared with the vegetative cells, to secure samples of cells in these various physiological states in adequate amounts. This calls for the cultivation of the test organisms under as rigidly controlled environmental conditions as possible. In the present work an endeavor was made to provide for these needs.

The organism chosen for this study, as in the previous work, was *B. mycoides.* Its adaptability for the purpose is well known and considerable important information on the biology of this organism has been accumulated by workers both here and abroad since the earlier papers of this series were printed.

This paper reports the observed effect on the production of spores by *B. mycoides* of the following environmental factors: (*a*) Oxygen supply; and (*b*) the food supply. Preliminary results obtained on the effect of dissociation on the mechanism of spore production are presented, and in order to indicate the generality of the results obtained with *B. mycoides* some brief comparative studies with other aerobic spore-formers are described.

RÉSUMÉ OF THE LITERATURE

In reviewing the significant studies on the physiology of spore production by aerobes, the following factors are discussed: (1)

The oxygen supply; (2) the temperature; (3) the hydrion concentration; (4) the relative importance of the food supply and the concentration of metabolic products in the medium; (5) the water content of the vegetative cells, and (6) the tendency to "dissociate."

In 1877, Cohn first demonstrated that *B. subtilis* forms spores, which were much more resistant to heat than are vegetative cells, and Koch (1877) showed that the cause of anthrax was the spore-forming organism, *B. anthracis*. These discoveries, the first of which dealt a finishing blow to the theory of spontaneous generation, and the second of which, independently made by Pasteur (1858), threw light on a practical problem and clarified a pathological mystery, naturally focused attention on these two spore-formers. The result was that most of the early physiological studies were confined to *B. subtilis* and *B. anthracis*, and, with the exception of the work of Blau (1906) and of Wund (1906), who studied the effect of temperature and of oxygen, respectively, on spore-formation in a number of spore-formers, it is not too much to say that critical work on the factors concerned in aerobic spore production has been limited to only six or seven species. In spite of this, general "explanations" of spore formation based on one or two species have been advanced more than once.

Cohn and Koch in the studies just mentioned were the first to note that the free access of air to cultures was essential for the formation of spores by the organisms they studied. This was confirmed, among others, by Buchner (1890), who, however, did not ascribe to oxygen any specific influence on spore production by *B. anthracis*; he believed the effect of oxygen was merely to make vegetative growth possible.

Schreiber (1896), working with *B. subtilis*, *B. anthracis*, and *B. tumescens*, concluded that oxygen was essential for the formation of spores, apart from its effect on vegetative growth. He found that in tubed liquid media at 30°C., *B. anthracis*, which is non-motile, and grows at the bottom of media, must have a layer of liquid no higher than 15 mm. over it if spore production was to occur; under the same conditions, *B. subtilis* and *B. tumescens* formed spores irrespective of the height of the liquid. However, when the cotton stoppers were replaced by cork, he found that *B. subtilis* must have a column of air at least 3 cm. high over the surface of the medium, and that *B. tumescens* required an air column at least 5 cm. high if spore production was to occur. With less than these amounts of oxygen, growth but not spore production took place.

Wund (1906), a student of A. Meyer, studied quantitatively the effect of the minimum, optimum and maximum oxygen concentration on spore germination, vegetative growth, and spore formation by a number of aerobic spore-formers, grown on glucose agar plates. He found, for example, the following data in the case of *B. mycoides*.

	OXYGEN PRESENT PER LITER, MGM.		
	Minimum	Optimum	Maximum
Spore germination	4.3	70	1,336
Vegetative growth	4.3	60	1,336
Spore formation	6.8	276	1,336

Among the organisms studied, the highest minimum figure for spore formation was 130 mgm. of oxygen per liter (*B. tumescens*, *B. pumulos* and *B. ruminatus*). The remaining minimal oxygen concentrations for spore production were much lower, ranging from 20 to 3 mgm. of oxygen per liter. Wund's data show interesting differences among the aerobic spore-formers studied as regards their relation to oxygen. In general, the minima for spore germination and vegetative growth (which were practically the same) were lower than the minima for spore production. In view of the fact that the addition of glucose to a medium enables many aerobic spore-formers to grow at much lower oxygen concentrations than are otherwise possible (see data of Eisenberg (1918) and others whom he cites), it is quite probable that the minimal oxygen concentrations obtained by Wund would have been quite different, probably higher, if glucose had been absent from the culture medium.

Holzmüller (1909) studying the physiology of five strains of *B. mycoides*, as well as of four closely related forms, also found that oxygen was essential to spore formation. He performed the interesting experiment of taking samples for microscopic observations at intervals from a broth culture in which spore formation could not go to completion because of an insufficient supply of oxygen. The time required for spore formation in the new environment varied inversely with the age of the broth culture. Thus, when the broth culture was two days old, twenty hours were required by the sample to form spores, but when the broth culture was fourteen days old, only half an hour was required for spore formation.

It seems fairly definite that the biochemical transformations leading to spore formation within the cells of aerobes demand oxygen for their completion. However, there are important differences among aerobic spore-formers as regards their relations to oxygen.

It was early discovered that spore production occurred only within a given range of temperature. Koch (1877) found that *B. anthracis* formed spores at 35°C. in twenty hours, but that this process required seven days at 16°C., while no growth or spore formation occurred below 15°C. According to Cohn (1877), *B. subtilis* neither grows nor forms spores above 50°C., but both processes occur at 47°C. Brefeld (1881) noted that below 5°R. (6.2°C.) *B. subtilis* grows slowly but does not form spores.

Schreiber (1896) observed the following minimal temperature relations: *B. subtilis* grew at 8°C., but formed spores only at 10°C.; *B. tumescens* grew at 10°C., but formed spores only at 11°C.; *B. anthracis* grew at 12°C., but formed spores only at 14°C. He has recorded the interesting observation that *B. anthracis* was injured and spore formation adversely affected by transferring abruptly from 37°C. to 18°C.

Migula (1897, 1904–07) confirmed the observation that spore formation did not occur at all temperatures at which vegetative growth took place. He found that although *B. megatherium* still grew at 12°C. or a little less, dividing about every three to four hours, spores were not formed at this temperature after six weeks. He obtained good colony development of *B. subtilis* after a week at 4 to 8°C. but found no spore production. According to him, the temperature limits for the growth of *B. anthracis* are from 10°C. to 43°C.; though the optimum for growth is 37°C. the optimum for spore formation is about 6° less. He found that the various strains of *B. anthracis* differed considerably with regard to the minimum temperature limit for spore formation, one forming spores at 16°C., while for another strain the lower limit was 24°C. Blau (1906) ascertained the maximal temperatures for spore germination and spore production for a number of aerobic spore-formers, and incidentally their optimal growth temperatures. The organisms were cultivated on glucose agar plates. It is interesting to note that *B. mycoides* neither grew nor formed spores at 35°C., only 5 degrees higher than its optimum for germination, growth and spore formation.[1] He further states that *B. cohaerens* formed no spores at 35°C., though it grew well at this temperature. Itano and Neill (1918–19) noted that *B. subtilis* completed the spore cycle at 37°C. in much less time than at 25°C.

Casman and Rettger (1931) found that the succinodehydrogenases of *B. mycoides* as well as those of a number of other spore-formers, were distinctly inhibited at the maximum temperatures of growth of these organisms. The heat labilities of the paraphenylene diamine oxidases of *B. cereus* and of *B. mycoides* varied considerably, but as a rule were found to approximate their maximum temperatures of growth. They made the interesting observation that *B. mycoides*, after exposure to the maximum temperature for growth for some time, lost the ability to grow at or near the surface of the agar but retained its ability to grow in the depths.

To summarize, it is clear that for some, and probably all, species of aerobic spore-formers the temperature range for spore production is narrower than the temperature range for growth; that is, growth may occur at temperatures too high or too low for the formation of spores. The temperature limits for both growth and spore formation vary not only with the species but with the strain or variety of spore-former.

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[1] "Strain differences" undoubtedly affect the level of the "maximum;" for example, Eisenberg (1918) reports strains of *Bacillus mycoides* which were "partly thermotolerant and partly thermophil," and which grew well at 55°. Holzmüller (1909) also found that the maximum temperature for spore germination, growth and spore formation varied according to the strain of *B. mycoides* used.

Only a small amount of work seems to have been done on the effect of the hydrion concentration of the medium on the process of aerobic spore production. The older work consisted chiefly in adding acidic or basic substances in various concentrations to a broth medium, and comparing the time required for spore formation with the time required by the same organism in a control medium. The general conclusion was reached that spore formation takes place not only in a definite range of temperature and of oxygen concentration, but also within definite limits of acidity and alkalinity.

Closely connected with such experiments are those in which the effect on spore production of adding available carbohydrates to the medium was studied. Though usually in such experiments the resulting acidity was not measured, it is apparent that the effect on spore production of adding glucose to a peptone medium was largely due to the degree of acidity which developed. Schreiber (1896), and later Gärtner (1903), made the important point that the effect of the addition of carbohydrate to the medium depends on the amount of available nitrogen present. For example, the addition of glucose to a medium retards growth and spore formation only when the available nitrogen supply is scanty.

Itano and Neill (1918–19) found that the optimum reaction for the germination of *B. subtilis* spores was pH 7 to 8, that the limits for growth were pH 4.2 and pH 9.4, respectively, and that germination and completion of the spore cycle at 24°C. and 37°C. occurred between pH 5 and pH 10. "The spores can germinate when the pH value is 10, although after germination the vegetative cells multiply only to a very slight extent and soon pass into spores. The slight growth and multiplication of vegetative cells in broth of pH 10 suggest that the formation of endospores in this medium must be caused largely by the unfavorable reaction of the medium rather than by the accumulation of end products." They state that the automatic adjustment of the medium by the growing organism seems to play a rôle in the completion of the spore cycle.

B. anthracis, according to Whitworth (1924), can grow in broth from pH 6.4 to more than pH 8.4, the optimum range being from pH 7.8 to pH 8.1 Delay in sporulation results from growth on excessively acid or alkaline agar. In view of his data, he regarded it as very probable that the hydrion concentration of soils plays an important rôle in the life of any *B. anthracis* spores present.

Although it is generally recognized that spore production by aerobes occurs within the acidity range whose limits are approximately pH 5 and pH 10, it is evident that relatively few studies deal with this point and that much more work needs to be done. The question of the effect of hydrion concentration on the velocity of spore production is of general biological interest. Investigations in this field deal essentially with the effect of hydrion concentration of the medium on the formation and storage of fat, protein, or glycogen in unicellular organisms. Such storage of reserve material generally precedes spore formation; the kind of material stored depends, of course, on the nature of the organism.

These three factors, oxygen concentration, temperature, and hydrion concentration, affect spore production, not only directly but indirectly, by their effects on growth. The closer cultural conditions are to the optimum with respect to these three environmental factors, the more rapid growth will be, and hence, after a given time period, twenty-four hours for example, the greater will be the utilization of the food supply and the greater will be the accumulation of metabolic products. The relative importance of these last two factors as a general cause of aerobic spore production is uncertain, for the reason that relatively few spore-formers have been studied from this point of view, and, furthermore, the attempt to disentangle these two factors—a very difficult task—has not on the whole been successful.

In 1877 and 1881, Buchner (1890) conducted experiments on the cause of spore formation by *B. anthracis*, which led him to conclude that "Die physiologische Ursache der Sporenbildung liegt in dem eintretenden Mangel an Ernährungsmaterial." Two types of experiments supported this view. He found that by transferring *B. anthracis* from one nutrient solution to another fresh nutrient solution at suitable intervals, vegetative growth without spore formation could be indefinitely maintained. This type of experiment is, however, equivocal as regards its interpretation, for, as Migula (1897) pointed out, failure of the vegetative cells to form spores could arise either from an excess of nutrient material or because transfer to a fresh nutrient solution was always made before the concentration of metabolic products became sufficient to cause spore formation.

The second type of experiment on which Buchner based his conclusion was his finding that transfer of well-nourished vegetative cells to distilled water (or 2 per cent sodium chloride solution) quickly led to complete spore formation (under suitable conditions of oxygen supply and temperature), while at the end of the same time vegetative cells placed in nutrient solution obtained from an old culture had not formed spores. In this experiment metabolic products seem to be excluded as a cause of spore formation by *B. anthracis*.

Buchner published these results in 1890 in a refutation of the theory of Lehmann (1890) as to the cause of spore formation by *B. anthracis*, which had appeared earlier in the year. Lehmann based his preliminary communication on data obtained by his student Osborne (1890). Osborne found that after increasing dilutions of broth cultures, inoculated with *B. anthracis*, had been aerated for a day and a half, growth and the absolute number of spores were directly proportional to the concentration of the medium. The number of spores was obtained by plating out the cultures after heating them for one hour at 65° to 70°C. Osborne concluded that spore formation is not favored in the slightest degree by dilute concentrations of the food supply.

Buchner criticised this experiment on the ground that it is not the absolute number of spores that is significant, but the number of spores relative to the number of vegetative cells. The degree to which a medium favors spore production, he believed, is given by the percentage of spores, which he called "the intensity of spore formation." This concept involves the idea of a velocity, which in turn involves the idea of a time interval. Buchner claimed that the time interval of a day and a half is too long and that twenty-four hours, even twelve hours under the most favorable conditions, is sufficient.

Osborne also plated *B. anthracis* on nutrient agar medium on which repeated crops of *B. anthracis* had previously been grown. Since, under these conditions, only sparse growth with slight spore formation was obtained, while numerous spores were obtained on fresh medium with an abundant food supply, he inferred that a depleted food supply is attended by only slight spore formation. He seems to have neglected entirely the factor of metabolic products in his interpretation of the experiment.

Stephanidis (1899), another student of Lehmann, found that the rapidity with which spores of *B. anthracis* are formed is greater on poorer media on which growth is sparse. He then attempted to determine the relative production of spores formed on plated agar media whose content of meat extract varied from 5 to 0.02 per cent. The temperature of incubation was 37°C.; the time of incubation was not stated. He did not determine the percentage of spores but seems to have averaged the number of spores per chain of cells, counting 10 chains that seemed representative. From his data he concluded that the relative number, as well as the absolute number, of spores increased directly with the concentration of the medium. It is difficult to see how his figures are comparable, for he found that growth was directly proportional to the concentration of the medium, and presumably the length of the chains of cells also increased with the concentration of the medium.

Turro (1891) believed that *B. anthracis* forms spores as a result of the accumulation of its products of metabolism. He concluded that the carbohydrate compounds of the cell are oxidized in the presence of atmospheric oxygen and metabolic products; the nitrogen compounds of the cell, which are the chief constituents of the spore, remain and, as end-products, condition spore formation.

Schreiber (1896) confirmed Buchner's finding that the vegetative state of *B. anthracis* could be indefinitely prolonged by periodic transfer to fresh media; this was also true of *B. subtilis* and *B. tumescens*. He also found that when well-nourished vegetative cells of these three organisms were placed in distilled water or in solutions of various salts, under favorable conditions of temperature and oxygen supply, spore formation promptly occurred. In his opinion, spore formation was induced by the sudden hindering of growth following previous good nutrition of the vegetative cells. He considered it improbable that the accumulation of metabolic products could be the cause of spore formation, since transfer of well-nourished vegetative cells to nutrient media in which spore formation had previously occurred again and again always resulted in a retardation of such formation.

On the other hand Migula (1897) stated that the induction of spore formation is mainly conditioned, not by the exhaustion of the food supply, but by the accumulation of metabolic products such as acids or alkalies. He believed that direct proof of this statement was afforded by the following experiment: When dry meat extract or dry peptone was added to a broth culture of *B. anthracis* shortly before spore formation, preparation for sporulation was not prevented though the food supply was greatly increased. If, however, at the same time, he added water with the dry nutrients no spore formation but resumption of growth took place; often, simply diluting with water had the same result. Decreasing the concentration of metabolic products permitted growth.

Holzmüller (1909), working with the "*B. mycoides* group," checked Buchner as regards the indefinite prolongation of the vegetative state by periodic transfer to fresh media and also with respect to his conclusion that the cause of spore formation was the exhaustion of the food supply. Vegetative cells in a good state of nutrition (two-day agar plate culture) were transferred to distilled water and to a fresh nutrient agar plate medium. After twenty-four hours the cells in distilled water had nearly all sporulated; there were no spores, but vigorous vegetative growth, on the nutrient agar plate.

Henrici (1928) made microscopic observations on spore formation by *B. cohaerens* on full strength nutrient and quarter-strength nutrient agar slants, each strength inoculated with a heavy and a light (1:50) spore suspension. Spore formation proceeded more rapidly in the quarter-strength agar media than in the full-strength media, regardless of the number of spores inoculated, and more rapidly in the heavily seeded than in the lightly seeded cultures. He inferred that the rate of spore formation is determined not by the concentration of cells alone but by the ratio of the population density to the concentration of nutrient material. He also found that spore formation by *B. megatherium* commenced practically at the point of inflection between the logarithmic growth phase and the resting phase, and that it then proceeded at a practically constant rate for some time, later decreasing in rate.

It will be noted that investigation of the relative importance of the nutrient value of the medium and of the concentration of metabolic products in the medium in inducing spore production has been confined to a few organisms only. That the relative importance of these two factors depends partly at least on the nature of the organism is shown by work reported in this paper. It is therefore highly desirable that many more organisms be studied from this point of view. The terms "food supply" and "metabolic products" are, of course, blanket terms. The cultivation of aerobic spore-formers on synthetic culture media seems not as yet to have been successful. The specific food factors essential for growth and spore formation are unknown. Likewise the metabolic products formed in complex media are largely unknown. The effect of the few known metabolic products, such as hydroxyl ion or ammonia ion, on spore production has not so far been investigated under controlled conditions.

Recently Daranyi (1927), who studied *B. anthracis*, *B. subtilis*, and *B. cylindrosporus*, has introduced a new factor in the study of spore formation. According to him, the most important influence favoring spore production is the decrease of the water content of the bacillus, an "entquellung" of the cell colloids. This occurs under natural conditions when the bacillus ages, "aging" involving a loss of water. Buchner's theory, he believes, holds to the extent that with the cessation of cell-division, occurring when the food supply becomes a limiting factor, the bacilli become older and thereby the water content decreases. Daranyi was able to induce an earlier spore production on agar by previously dehydrating the spores used as inoculum; for example, the spores of a virulent strain of *B. anthracis* that had been dried for two days in a desiccator produced 70 per cent spores after eighteen hours incubation on a freshly prepared agar slant, while on the control, inoculated with an eighteen-hour agar slant growth, only 1 per cent spores were produced after eighteen hours incubation at the same temperature.

That the same kind of organism is not constant in its response to environmental conditions as regards spore production, and that any given response depends, partly at least, on its previous cultural history, are shown by the work of investigators who have succeeded in obtaining permanently asporogenous strains from sporogenous strains of aerobic spore-formers.

Lehmann (1888) found a fully virulent but asporogenous strain of *B. anthracis* among a number of old cultures at the Berlin Hygienic Institute. Behring (1889) obtained two asporogenous strains of *B. anthracis* by growing them for several months on gelatin agar medium containing, in the one case, 1 per cent hydrochloric acid, and in the other, rosolic acid. Roux (1890) obtained asporogenous strains of *B. anthracis* by cultivating it in broth containing small amounts of phenol (less than 1 to 1666). This procedure was confirmed by Migula (1897) for non-motile, but not for motile, spore-formers. Phisalix (1892) cultivated *B. anthracis* at 42°C. to get it in the asporogenous state. Nadson and Adamovic (1910) cultivated *B. mycoides* on a meat-peptone gelatin medium; the old liquefied gelatin medium was added after sterilization to an equal volume of an agar or bouillon medium containing constituents, with the exception of water, in double amount. Grown on such a medium, *B. mycoides* changed, they reported, beyond recognition; the cultures resembled those of Actinomycetes. The properties of gelatin-liquefaction and sporulation were lost.

The loss of the property of spore production seems to be part of the large and complex problem of microbic dissociation. No attempt will be made to present a complete consideration of the work dealing with dissociation phenomena in aerobic spore-formers. A few illustrations from recent literature, however, to make clear the point that dissociation profoundly affects aerobic spore-formers, will be presented.

Löhnis and Smith (1923), studying the genus *Azotobacter*, have described two of the seven cell types found as small sporulating rods and large sporulating rods. The former type is identical with *B. terminalis* Mig., *B. fusiformis* A.M. et Gottheil and in the case of *A. chroococcum* and *A. Beijerinkii*, with *B. pumilis* A.M. et Gottheil. The latter form, which produced white, yellow and brown colonies, was identical with *B. luteus* Baker et Smith, *B. petasites* A.M. et Gottheil, *B. malabarensis* Löhnis et Pillai and *B. danicus* Löhnis et Westermann. They state that all cell types were transformed into each other.

Cunningham (1931) stated that he had obtained two aerobic spore-formers as variants from the anaerobic spore-former, *B. saccharobutyricus* von Kelcki (*Clostridium butyricum Prazmowski*). One of these variants was identical with *B. cereus* Frankland and the other corresponded to *B. sphaericus* Meyer.

Haag (1926) reported a study of a culture named *Bac. viridiglucescens* Sack. He found that after this organism had been incubated two to three weeks on agar plates, each grayish-yellow round colony had become surrounded with a white marginal growth. These secondary colonies contained small oval spores and large oval or round vegetative cells. Streak cultures of the marginal growth showed thin rods (originating from the spores) and thick, plump oval cells. The thin rods, which showed good spore formation, were identical with *B. mesentericus* in all points. The thick, plump organism corresponded to *B. megatherium* De Bary. It exhibited extremely slow spore formation.

Two very interesting studies of dissociation in *B. mycoides* have been made by Oesterle and Stahl (1929). Their results (independently conducted but reported together) will be considered separately.

Oesterle studied three strains of *B. mycoides* in three media: (1) germ-free soil extract, (2) germ-free filtrate of "Faülflüssigkeit," and (3) broth exposed to sunlight and also to ultraviolet light. Typical rods of *B. mycoides*, incubated for several months in the first two of these media, generally autolyzed; numerous small granules (Kugelformen) were present. These, after some passages on agar and in broth, grew to rods, which afforded smooth, soft agar colonies (S-form). From these colonies there appeared, under conditions not yet established, typical *B. mycoides* outgrowths. Typical *B. mycoides* could be cultivated from these outgrowths. The S-form was motile, growing singly or in groups of two, and formed spores.

Exposure of broth cultures of typical *B. mycoides* to direct sunlight for a week resulted in the formation of granules which passed through kieselguhr filters and reverted after several months to typical *B. mycoides* after passing through a succession of intermediate forms.

When broth cultures were exposed to dosaged illumination with ultraviolet light, typical *B. mycoides* was transformed to granules, thread forms, and small Gram-negative rod-like gonidia. These three forms after a number of transfers eventually reverted to typical *B. mycoides.*

Stahl found that typical *B. mycoides* disintegrated to filterable forms under the influence of sodium chloride and sodium chloramine Heyden and of bichloride of mercury. The atypical forms thus produced reverted by way of a coccus stage and smooth colony forms to typical *B. mycoides.* He stated that "the degeneration forms noted in the literature many times are in part forms capable of development from the developmental cycle of *B. mycoides.*" In his opinion, the species *B. effusus*, *B. olfactorius*, and *B. nanus*, described and pictured by Holzmüller, belong with great probability to the developmental cycle of *B. mycoides.*

Nyberg (1929) cultivated a number of strains of *B. mycoides* in liquid media for two months; from these old cultures a number of interesting variants were obtained. These variants, as well as those from other spore-formers, showed in colony form and cultural characteristics, all transitions between *B. mycoides*, *B. mesentericus*, and *B. subtilis*.

Though the effect of dissociation on the mechanism of spore formation is not specifically considered in these reports, it is very plain that spore production in general is profoundly affected by dissociation changes. It is outstanding that during the transition from the "typical" to an atypical form there is little or no spore production. Apparently a variant from the typical form may or may not be a spore-former, though it may later change into a spore-former. It seems plain from recent work of the type here cited that studies of the biology of old cultures will clarify relations in the classification of spore-formers, which are at present obscure.

METHODS AND APPARATUS

The culture vessels used in the beginning of this work consisted of test tubes of 75 cc. capacity, fitted with two-hole rubber stoppers, carrying straight inlet and outlet tubes. (For convenience this type of aeration apparatus will be referred to as "straight culture vessel.") Compressed air was passed through a solution of $KMnO_4$ to a manifold, from which connections were made through needle valves to the culture vessels. The air pressure was regulated by a mercury head, 2½ to 3 inches high. With this arrangement difficulty was experienced in securing uniform, uninterrupted bubbling, and as a result marked variations in the amount of growth and in spore production after twenty-four hour's aeration were often noted.

Early in this work it was realized that a more uniform environment for the cultivation of bacteria could be secured by aeration in liquid media than by the use of solid substrates. The advantages to be derived have been discussed by the writers in a previous publication (Magoon and Brunstetter (1930)), in which a culture vessel designed to secure efficient aeration and based on the principle of the gas scrubber has been described in detail. (This second type of aeration apparatus will be designated as the "spiral culture vessel.")

It should be emphasized that with both forms of apparatus contamination was prevented not only by passing the air through a solution of $KMnO_4$ but also through a cotton plug before it entered the culture media.

As a consequence of the uniformity of distribution of food material and metabolic products in efficiently aerated cultures, quantitative counts of the percentage of spores formed in a given medium after twenty-four hours may be undertaken without the variability of results that may be expected in unaerated liquid media or on plates or slants of agar media. The percentage of spores was considered an adequate measure of the "intensity factor" of the spore-producing power of a given cultural environment, which is what is desired. Consequently, the number of spores of a given culture, which may be regarded as a measure of the "capacity factor" of its spore-producing power, was not determined.

The strain of *B. mycoides* employed for most of this work was isolated from Texas soil in 1922 and was chosen because of the high thermal resistance of its spores. Several single cell cultures from this strain were made by means of the Chambers micromanipulator and were used as sources for subsequent transplants in this work. This strain gave cultural reactions in all respects identical with those regarded as typical of *B. mycoides* Flügge. The other strains used were isolated in 1931 from soils secured from various parts of the country. The strains are designated by the names of the experiment stations from which they came.[2]

A number of unsuccessful attempts were made to secure a synthetic medium on which *B. mycoides* would grow vigorously. Consequently, a peptone medium, Difco bacto-peptone, was used throughout the investigation.

All incubations were made at 30°C.

EXPERIMENTAL

The effect of aeration on the growth of B. mycoides in 2 per cent peptone solution

Early in the investigation it was found that aeration of peptone cultures of *B. mycoides* greatly increased the amount of growth obtained in twenty-four hours. As an illustration, the following experiment is presented: Four spiral culture vessels were sterilized and 70 cc. of sterile 2 per cent peptone were transferred to each vessel. After inoculating each medium with 0.1 cc. of a spore suspension of *B. mycoides*, the vessels were connected to the aeration apparatus and vigorously aerated for a minute to secure an even distribution of the inocula. The air supply was then shut off from two vessels and in the other two vessels aeration was adjusted at the rate of 60 to 70 bubbles per minute. After twenty-four hours, 30 cc. from each culture were centrifuged until the supernatant liquid was clear and the volumes of the sediments were read. The average for the controls was 0.06 cc.; the average for the aerated cultures was 0.60 cc.

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[2] These soils had been collected in 1918 and were stored in the laboratory for thirteen years.

The inference from this result, of course, is that in an aerated medium of about 0.5 per cent peptone solution, exhaustion of the food supply will occur much sooner than in a medium of the same composition unaerated, and also that there will be a correspondingly greater accumulation of metabolic products. The great advantage of aerating a liquid medium is that growth is not then limited by an insufficient oxygen supply, with the result that the effect of the factors of food supply and metabolic products on spore production may be much more profitably studied. Another advantage, perhaps no less important when dealing with an organism like *B. mycoides*, which grows in unstirred liquid media almost entirely on the surface as a pellicle, is that bubbling air through the medium prevents localized growth, so that the concentration of the food supply as well as that of products of metabolism is uniform throughout the medium. The use of aeration is, of course, not new in the analysis of the physiology of aerobic spore formation; it has been used, for example, by Buchner, Osborne, and by Stephanidis. So far as the application to aerobic spore-formers like *B. mycoides* goes, its importance in studying spore formation can not be over-emphasized.

The effect of the concentration of peptone in unaerated cultures on spore production

Peptone solutions of the following concentrations were prepared, tubed, and sterilized: 2, 1, 0.5, 0.25, and 0.12 per cent. After inoculation with equal volumes of a spore suspension of *B. mycoides* they were incubated for forty-three hours; smears were then made, using pellicle material only, for determinations of the percentages of spores, using three preparations from as many tubes for each concentration.

It was found that less than 1 per cent of spores was produced under these conditions in any concentration of peptone. This result, often repeated, shows that the effect of the peptone concentrations on spore production is obscured under these conditions.

The effect of the concentration of peptone on spore production in aerated cultures of B. mycoides

Peptone solutions were made in the following concentrations and sterilized: 5, 2, 1.5, 1.0, 0.75, 0.50 and 0.25 per cent. After sterilizing the straight culture vessels, 25 cc. of each solution were transferred to them; the media were then inoculated with equal amounts of spores of *B. mycoides* from a sand culture. The culture vessels were then connected to the aeration apparatus and vigorously aerated for twenty-four hours. Duplicate tubes of each concentration were employed. After twenty-four hours the tubes were disconnected, the nature of the growth noted, smears prepared for microscopic examination, and 15 cc. of the cultures centrifuged for ten minutes. At the end of this time, the growth was completely thrown down. The volumes of the sediments obtained were then noted. This means of recording growth is relatively crude, yet it nevertheless gives an approximate measure of the yield of cells obtained for a given concentration. Plate counts or direct microscopic counts do not give accurate results under these conditions because growth occurs in clumps whose size increases with the concentration of the medium. The results of four such runs, in which there were duplicate cultures for each concentration, are presented in table 1 and figure 1.

It will be noted that the peptone concentrations have been expressed not in the form of percentages, but as milligrams of peptone initially present in the medium. This gives the total amount of nutrient present in the environment and allows the results to be compared with subsequent runs, in which the spiral culture vessels were used, each containing 70 cc. of peptone solution.

The figure shows clearly, first, that with an adequate oxygen supply the amount of growth was directly proportional to the amount of peptone present in the culture (a fact which had been noted by previous investigators); second, that the number of spores relative to the number of vegetative cells increased as the amount of peptone present in the culture decreased.

TABLE 1

Effect of the amount of peptone in the medium on growth and spore production by B. mycoides after aeration for twenty-four hours

RUN NUMBER	MILLIGRAMS OF PEPTONE INITIALLY PRESENT IN 25 CC. OF MEDIUM														INOCULA—NUMBER OF SPORES
	1,250		500		375		250		187		125		63		
	Yield	Spores	Yield	Spores	Yield	Spores	Yield	Spores	Yield	Spores	Yield	Spores	Yield	Spores	
	cc.	*per cent*	*cc.*	*per cent*	*cc.*	*per cent*	*cc.*	*per cent*	*cc.*	*per cent*	*cc.*	*per cent*	*cc.*	*per cent*	
I		0	0.4	0	0.3	6	0 2	80	0.2	95	0.1	95	0.05	100	9,500,000
		0	0.4	23	0.4	40	0.2	50	0 2	90	0.1	90	0.05	95	
II		0	0.4	0	0.25	35	0 2	42	0.15	45	0.1	99			4,900,000
		0	0 4	0	0 25	52	0.2	60	0.15	80	0.1	90			
III	0.6	0	0.4	0	0.4	32	0.2	78			0 1	95	0 08	99	6,200,000
	0.6	0	0.4	41	0.4	56	0.2	70			0.1	99	0.04	99	
IV	0.6	0	0.4	0	0.25	9	0.2	80			0.1	52	0.05	90	6,900,000
	0.6	0	0.4	0	0.35	55	0.2	63			0.1	90	0.05	95	
Mean..	0.6	0	0.4	8	0.33	36	0.2	65	0.13	78	0.1	88	0.05	98	

The figures for the percentage of spores often vary considerably for duplicate cultures containing the same amount of peptone. This was generally due to the fact that the rate of aeration in one of the cultures was by accident markedly slower than in the duplicate culture. This slower aeration was usually accompanied by a lower production of spores.

In a second series of runs, the rate of aeration was varied as well as the concentration of peptone, since previous observations had demonstrated the great importance of the oxygen supply, as a factor not only in the production of growth but of spores in a peptone solution of a given concentration.

The inoculum for the first run came from the ninth daily transfer in aerated 4 per cent peptone solution; the inoculum for run 2, from the eleventh transfer, and the inoculum for run 3, from the thirteenth daily transfer. In this series of runs the spiral culture vessels were used, each containing 70 cc. of peptone solution; the experiment set up was such that the rate of aeration could be accurately maintained throughout the twenty-four hours. The concentrations of peptone used were 1.0, 0.5, 0.25, 0.12 and 0.06 per cent. Each tube was inoculated with 0.1 cc. of a 4 per cent peptone culture aerated for eighteen hours; the cells were all in the vegetative state. After incubation all the tubes were adjusted to aerate approximately at the same rate. At the end of twenty-four hours, smears were prepared from each tube for the determination of the percentage of spores and for cell counts.

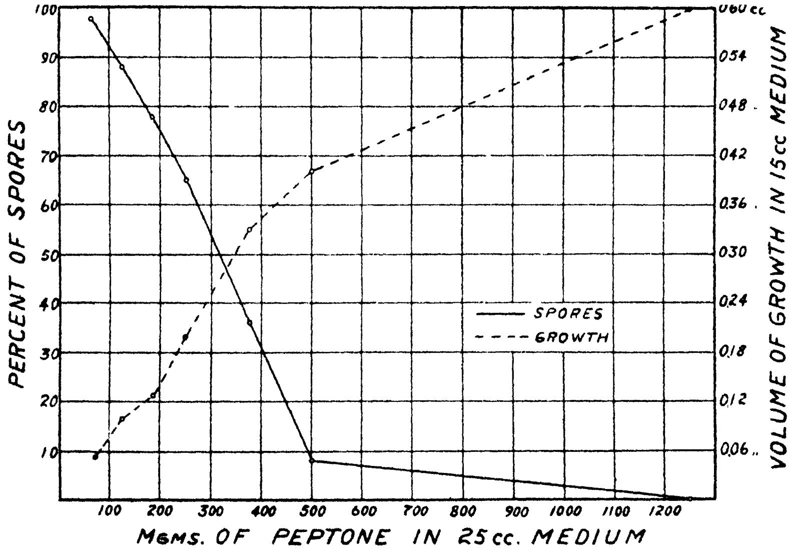

FIG. 1. PERCENTAGE OF SPORES AND VOLUME OF GROWTH OF *Bacillus mycoides* IN AERATED PEPTONE SOLUTIONS AFTER TWENTY-FOUR HOURS' INCUBATION AT 30°C. AS FUNCTIONS OF THE AMOUNT OF PEPTONE PRESENT IN THE MEDIUM

In this way three successive runs were made in which the degree of aeration was 60, 150, and 250 to 300 bubbles per minute, respectively. The concentration of peptone, as in the first series of runs, is expressed in milligrams of peptone present in the medium (in this case 70 cc.).

The nature of the vegetative growth of *B. mycoides* changes after repeated transfer in aerated broth or peptone media. As previously stated, growth in peptone solutions inoculated with spores occurs in clumps. If such a clump is transferred into a fresh peptone or broth solution, growth after four or five daily transfers no longer is present in the form of clumps but is uniform throughout the medium, so that a representative sample for microscopic cell counts may easily be taken.

TABLE 2

Growth and spore production by B. mycoides as functions of the amount of peptone in the medium and the rate of aeration: incubated twenty-four hours

RUN NUMBER	RATE OF AERATION BUBBLES PER MINUTE	MILLIGRAMS OF PEPTONE INITIALLY PRESENT IN 70 CC. OF MEDIUM									
		700		350		175		88		44	
		Spores	Number of cells per cubic centimeter	Spores	Number of cells per cubic centimeter	Spores	Number of cells per cubic centimeter	Spores	Number of cells per cubic centimeter	Spores	Number of cells per cubic centimeter
		per cent		*per cent*		*per cent*		*per cent*		*per cent*	
1	60	0	137,500,000	26	70,300,000	41	65,500,000	55	25,300,000	93	20,100,000
2	150	11	128,100,000	36	117,200,000	61	40,900,000	78	32,700,000	94	17,900,000
3	250–300	24	171,900,000	61	85,900,000	76	59,700,000	94	39,700,000	95	15,600,000

Inocula (number of vegetative cells per cubic centimeter): Run 1, 29,700,000, Run 2, 75,700,000; Run 3, 11,000,000.

The data in table 2 generally checked the figures of the first series of runs with respect to the increased growth obtained with an increased food supply, and also the increase in the percentage of spores with a decreased food supply.

The fundamental importance of the oxygen supply in spore production by *B. mycoides* is demonstrated by the increased spore production with an increased rate of aeration for any given peptone concentration. This is illustrated in the curves of figure 2.

Of particular interest are the slopes of all three curves for spore production, comparing the cultures containing 88 and 44 mgm. of peptone, respectively. It will be seen that where the rate of aeration was 60 bubbles per minute, the percentage of spores dropped sharply; where the rate of aeration was 150 bubbles per minute, the percentage of spores dropped less sharply, and where the aeration was 250 to 300 bubbles per minute the drop was only slight. The slopes of the curves for spore production at the higher concentrations show much smaller differences, the percentage of spores varying with the amount of peptone present but the slopes remaining roughly the same.

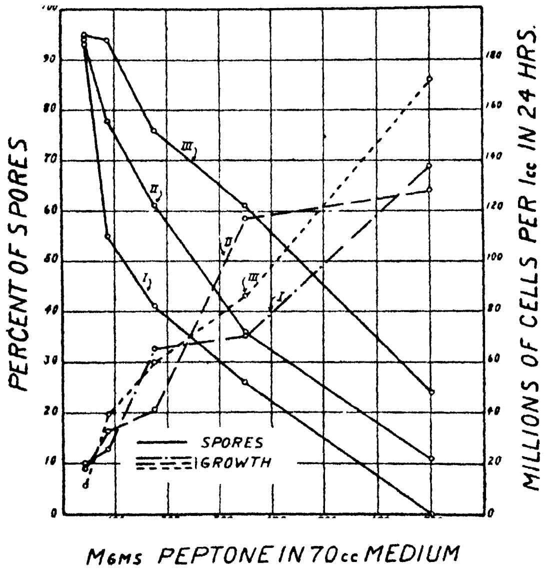

FIG. 2. PERCENTAGE OF SPORES AND VOLUME OF GROWTH OF *Bacillus mycoïdes* IN AERATED PEPTONE SOLUTIONS AFTER TWENTY-FOUR HOURS' INCUBATION AT 30°C. AS FUNCTIONS OF THE AMOUNT OF PEPTONE PRESENT IN THE MEDIUM AND THE DEGREE OF AERATION

I, aeration at the rate of 60 bubbles per minute; II, aeration at the rate of 150 bubbles per minute; III, aeration at the rate of 250 to 300 bubbles per minute.

The interpretation of these growth and spore production curves seems to be that the amount of growth is determined primarily by the amount of food present; the greater the nutrient supply the more abundant the growth, at least within the limits employed in the present experiments. As indicated by the intersections of the growth curves, the effect of the rate of aeration on the growth obtained is slight, if present, and is within the experimental error of estimating growth. In the case of spore production, on the other hand, two factors are clearly operative, namely, the food supply and the degree of aeration. With increased food supply the percentage of spores produced is progressively decreased, but this decrease is offset to a marked degree by an increase in the rate of aeration. If high spore production is desired the degree of aeration must be increased where higher food concentrations are used.

The importance of the rate of aeration can be demonstrated strikingly by the addition of methylene blue, so as to make a 0.005 per cent solution, to twenty-four-hour aerated cultures of *B. mycoides.* When aeration is discontinued, reduction of the dye with discoloration of the medium occurs in a few minutes. The rate of aeration required to restore the color of the medium throughout depends on the amount of growth, which in turn depends mainly on the amount of peptone in solution. It has been found, for example, that an aeration rate of 100 bubbles a minute was necessary to reoxidize completely the methylene blue in a culture aerated for twenty-four hours in 3 per cent peptone solution.

It will be noted that the size of the inoculum was variable, not only from run to run, but within a given run. Although in a given run the same volume of suspension of spores or vegetative cells was employed to inoculate solutions of decreasing amounts of peptone, the number of cells per milligram of peptone doubled as the amount of peptone in the medium was halved. Thus, in Run 1 of the second series, where the inoculum was 29,700,000 cells in 70 cc. of medium, there were 42,000 cells per milligram of peptone in the 1 per cent peptone solution, but in the 0.5 per cent peptone solution, which received the same volume of cell suspension, there were 85,000 cells per milligram of peptone. The question then arises as to what extent the relative spore production after twenty-four hours aeration was influenced by variation in the number of organisms per milligram of peptone initially present.

The writers believe that under the experimental conditions employed, the ratio of the population density to the food supply at the beginning of a run is of little or no importance. For a given concentration of peptone, aeration accelerates growth to such an extent that by the end of twenty-four hours the number of cells per milligram of peptone is practically constant and further growth is restrained, due either to the exhaustion of the food supply or to the accumulation of metabolic products, depending on the concentration of the peptone.

An examination of figure 2 shows that any effect of a changed ratio of cell population to the food supply is of much less importance than the effect of a change in rate of aeration. The amount of growth in media containing 700 mgm. of peptone, for example, varied inversely with the inocula. The second run, which received the greatest inoculum, showed the greatest amount of growth in only two of the five concentrations.

However, the importance of the size of the inoculum on the relative number of spores formed in a series of aerated peptone solutions of decreasing concentration would very probably be outstanding if the runs were to be ended at twelve or eighteen hours, before the end of the logarithmic period of growth.

Previous investigators have pointed out that the absolute number of spores increases with increased concentration of medium. When the percentages of spores in table 2 are multiplied by the number of cells per cubic centimeter, a rough measure of the total number of spores is obtained. It will be noted that the maximum number of spores per cubic centimeter produced in Run 1 was in the medium containing 175 mgm. of peptone. Increasing the amount of peptone resulted in a decrease in the absolute, as well as the relative number of spores. Similarly, in the second and third runs, the maximal absolute numbers of spores were in the medium containing 350 mgm. of peptone. With an increase in food supply above this amount the absolute number of spores decreased.

The effect of extending the time of aeration on spore production

In the preceding experiments the peptone cultures had been incubated for twenty-four hours only. This short incubation period leaves unanswered the interesting question as to whether prolonged aeration at a rapid rate would eventually bring about complete spore production in the higher concentrations of peptone, where heavy growth is obtained. This question is of importance from the standpoint of obtaining spores in quantities sufficient for chemical analysis.

The experiments performed in this connection are summarized in table 3.

TABLE 3

Spore production by B. mycoides after long-continued aeration in peptone solutions

MILLIGRAMS OF PEPTONE IN MEDIUM (70 cc.)	RUN STARTED	AERATION PERIOD	STRAIN OF B. MYCOIDES	SPORES
		hours		*per cent*
2,800	October 13, 1930	264	Texas	0
1,400	December 11, 1930	144	Texas	6
1,400	January 16, 1931	22	Texas	0
		70		1
700*	December 17, 1930	24	Texas	0 0
		48		0 2
		72		11 16
		96		3 10
		168		7 18
		240		41 85
1,400	March 19, 1931	192	South Carolina	3
		192	Oklahoma	3
		192	Virginia	9
		192	Maine	17
		192	West Virginia	31

* The figures for spore production here represent counts on duplicate tubes. All spore counts are averages, each based on counts of two smears.

The fact that no spores were observed at any time in eleven days in the medium containing 2800 mgm. of peptone (4 per cent) might mean that the cell population, though normal, had not yet exhausted the food supply or that metabolic products had injured the cells so that they were incapable of sustained growth or of forming spores. That the latter was the case is shown, first by the marked decrease in the number of bacilli after the third day of aeration and a corresponding increase in the amount of granulation of the cells that had occurred, and, second by the occurrence of dissociation. The culture was plated out on the eleventh day; in addition to typical *B. mycoides* the plates showed a variant, designated M 1 for convenience, which grew in compact, non-mycelioid colonies with sprawling out-runners of growth that curved at the end in a manner similar to typical colonies of *B. mycoides*. The growth, unlike that of typical *B. mycoides*, was non-adherent to the medium. This variant apparently is similar to that found by Nyberg (1929) (see Abb. 5, I–c, and Abb. 7, III–c).

DISSOCIATION AS A FACTOR IN SPORE PRODUCTION BY B. MYCOIDES

It was pointed out in the introduction that there was abundant evidence in the literature that certain aerobic spore-formers may undergo profound changes in morphology and colony form. However, the degree to which the mechanism of spore production is thereby altered is only scantily and indirectly indicated by past investigations.

The writers have found that dissociative changes play a very important part in the response of *B. mycoides* to aerated peptone solutions of decreasing concentrations. A culture undergoing dissociation may be totally unresponsive to the concentration of the medium as regards spore production. This is illustrated by the following experiment: A spore suspension was prepared from an old agar slant culture of the Texas strain of *B. mycoides*. It should be explained that this culture was only a few transfers removed from a culture obtained by the isolation of a single spore of *B. mycoides*. The suspension was used to inoculate 4 per cent peptone, which was aerated for twenty-four hours. Daily transfers in 4 per cent peptone were made, with the exception that transfer No. 6 was aerated for sixty-five instead of twenty-four hours (the culture was negative for spores and showed rather extensive autolysis). At intervals the twenty-four-hour culture was used to inoculate a series of peptone concentrations (in the spiral culture vessels); after twenty-four hours' aeration the cultures were then examined for spore production.

The results are given in table 4.

When the stock culture had been transferred every twenty-four hours for four days, subcultures in aerated peptone solutions of decreasing concentrations showed subnormal spore production; after the seventh transfer of the stock culture, subcultures in dilute aerated peptone solutions failed to produce spores.

TABLE 4

Effect of decreasing concentrations of peptone on spore production by B. mycoides

RUN NUMBER	NUMBER OF TRANSFERS IN AERATED 4 PER CENT PEPTONE SOLUTION	MILLIGRAMS OF PEPTONE INITIALLY PRESENT IN THE MEDIUM				
		700	350	175	88	44
		Percentage of spores after twenty-four hours' aeration				
1	4	0	2	0	43	53
2	7	0	0	0	0	0
3	10	0	0	0	0	1
4	14	0	0	0	0	0

In the first run the low spore production can be explained by the low rate of aeration (20 to 40 bubbles per minute) with the possible exception of the culture containing 44 mgm. of peptone; in this case aeration was started at 30 bubbles per minute, but by the following morning had risen to 180 bubbles per minute. In the second and fourth runs the rate of aeration was 60 bubbles per minute; the third run was aerated at the rate of 150 bubbles per minute.

The stock culture was plated out after the ninth transfer in 4 per cent peptone. The majority of the forty-eight-hour colonies on nutrient agar (see fig. 3) were definitely "atypical"; that is, they did not show the coarse mycelioid growth characteristic of *B. mycoides*; they corresponded to the "M 1" type of variant already noted. A number of small, smooth, white colonies were also present.

The different types of colonies showed an abundance of spores after four days' incubation, indicating that the variants produced were not asporogenous. Microscopic examination of the smears of the second, third and fourth runs showed that in general the cells presented a finely granular appearance and took the stain lightly. It will be remembered that in experiments summarized in table 2, the peptone solutions were inoculated with a culture of *B. mycoides* which also had been transferred daily in 4 per cent

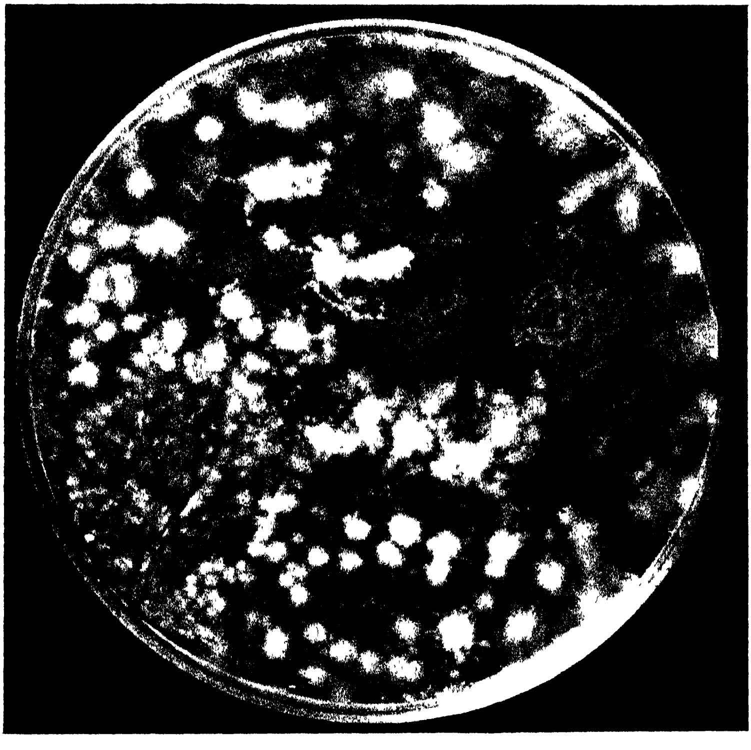

Fig 3 An M_I" Type Variant Obtained from Typical *Bacillus mycoides* after 9 Consecutive Transfers in 4 Per Cent Peptone Solutions

Plate incubated for forty-eight hours at 30°C peptone solution; in the case of the first run, the inocula came from the ninth daily transfer. However, no dissociative phenomena or lowered spore production were noted.

The writers believe that the different results obtained after repeated transfer in 4 per cent peptone in these two series of runs illustrate the different resistance to dissociative changes displayed by different cultures of the same organism. In the one case, repeated transfer in aerated 4 per cent peptone solution did not affect *B. mycoides*; in the other case, such cultivation induced dissociation which profoundly affected the mechanism of spore production. The reason for this difference is at present unknown. The importance of understanding this difference is obvious, as it is highly desirable in a chemical study of the thermal resistance of spores to cultivate the test organism under conditions which insure a stable strain.

TABLE 5

Spore production by the M 2 variant of B mycoides

EXPERIMENT NUMBER	AERATED FOR	MILLIGRAMS OF PEPTONE PRESENT IN THE MEDIUM				
		1 400	700	350	175	87
		Percentage of spores after aeration				
	hours					
I	24	36	29	39	9	11
II	24	10	10	0	4	0
	48	17	8	3	28	1
	72	14	7	1	38	8

A smooth variant of *B. mycoides* frequently encountered in our studies, has the following characteristics: It is a small, slender bacillus forming spores centrally, possessing active motility, growing singly or in short chains of two or three cells and forming on nutrient agar white or yellowish white, smooth, slightly raised colonies, at first round and entire but later forming an indented border zone. This variant, designated for convenience the "M 2" type, is similar in many characteristics to a smooth type described by Oesterle and Stahl (1929).

The results on the mode of spore production by this variant grown in aerated peptone solutions of varying concentrations are given in table 5.

The results of these two runs, while not checking closely, and, as far as the second run is concerned, lacking regularity, show clearly that, contrary to the results obtained with the typical, or rough, type of *B. mycoides*, there is here a decrease of spore production with a decreased concentration of medium. This smooth type of *B. mycoides* shows increased growth with increased concentration of peptone, so that in higher concentrations of peptone there is a greater concentration of metabolic products. In this case, then, metabolic products instead of hindering spore production seemed to favor it.

SPORE PRODUCTION BY AEROBES OTHER THAN B. MYCOIDES

In order to learn whether the results obtained with *B. mycoides* were general, a number of other aerobic spore-formers were studied.

As in the case of *B. mycoides*, peptone solutions of various concentrations were prepared, sterilized and transferred to spiral aeration vessels. After aeration for twenty-four hours at 30°C., at a rate of about 60 bubbles per minute, the vessels were disconnected and smears were prepared from each culture. Quantitative determinations of spore production were made from each smear.

No spores in any concentration of peptone were found in aerated cultures of *B. aterrimus*, *B. brevis*, *B. circulans*, *B. mesentericus*, *B. fluorescens*, *B. globigii*, *B. laterosporus*, *B. lacticolus*, *B. panis*, or *B. subtilis*. No growth after twenty-four hours' aeration was noted in the cultures of *B. laterosporus* and *B. panis*; fair growth, without spore formation, occurred in the subsequent twenty-four hours' aeration of these cultures.

B. tumescens also showed poor growth. After twenty-four hours' aeration of a series of peptone cultures ranging in concentration from 2 per cent to 0.03 per cent, the only growth that occurred was in the 1 per cent peptone culture, which contained 64 per cent spores. The media, with the exception of the 2 per cent and 1 per cent peptone cultures, were reinoculated and reaerated; after the next twenty-four hours' aeration only the 0.5 per cent peptone culture showed visible signs of growth. No spores were present in this growth. After a further aeration for twenty-four hours the 0.06 per cent peptone culture showed fairly heavy growth, while the 0.25 per cent and 0.03 per cent peptone cultures showed but slight growth. No spores were found in these cultures.

In a second experiment, medium turbidity after twenty-four hour's aeration was found only in the 2 per cent and 1 per cent cultures. The other cultures (4, 0.5, 0.25, 0.12 and 0.06 per cent peptone) showed no visible signs of growth. There were no spores in the 2 and 1 per cent peptone cultures.

In the case of *B. sphaericus*, the first experiment was negative. In the second experiment, 0.8 per cent spores were found in the 4 per cent peptone culture, 2.8 per cent spores in the 2 per cent, and 0.7 per cent spores in the 1 per cent peptone cultures. Cultures having a lower percentage of peptone were negative for spores.

Two experiments with *B. simplex* were negative except that two dilute concentrations of peptone gave 4 and 6 per cent spores, respectively.

The data obtained in experiments dealing with organisms which gave positive results are presented in table 6.

It will be noted that in quite a few instances the experimental results do not check. This lack of concordance, together with finding more than one type of bacillus in the same peptone cultures, encountered in the cases of B. *asterosporus*, *B. adhaerens*, *B. globigii*, *B. lactimorbus*, and *B. subtilis*, plus the evidences in the literature, is the basis of the writers' belief that with most if not all the spore-formers studied, the relation between the available food supply and the percentage of spores after twenty-four hours' aeration is complicated by the factor of dissociation. If cultures in the process of dissociation are unable to produce spores, a possible explanation of the negative results obtained with a number of spore-formers is that cultures of these organisms were, under the experimental conditions employed, undergoing dissociation. At the time these comparative tests were made, the importance of dissociation as a factor in spore production was not realized. As a result, clear-cut evidence of dissociative changes in cultures of these organisms was neither sought nor obtained.

TABLE 6

Relative spore production as a function of the amount of peptone present in the medium; cultures aerated for 24 hours

ORGANISM	EXPERIMENT NUMBER	MILLIGRAMS OF PEPTONE INITIALLY PRESENT IN THE MEDIUM																		
		2,800	1,500	1,400	750	700	500	375	350	250	183	175	125	88	62	44	38	31	16	8
		Percentage of spores																		
B. adhaerens	I						0			0			0		7			1	59	
	II	0		3		69			72			80		81		72				
B. albolactis	I						0								95				95	86
	II	0		16		97			93			93		98		97				
B. cereus	I						0			90–100			90–100		90–100			90–100		
	II	0		15		31			99			96		97						
B. cohaerens	I						0			1			1		3			2	12	
	II	0		0		0			0			0		0		0				
B. fusiformis	I												66		58			23	68	3
	II	79		85		84	79		83	72		69		64		35				
B. graveolens	I						27			18			16		12			13		0
	II	3		26		4			0.1			0 6		2		37				
B. lactis	I	79		5		7			14			27		15		13				
B. malabarensis	I		0		0			61			99					100				
B. megatherium	I		0		74						36		50				37		13	
	II	0		0		0		0						0		0				
	III	7		26		54				46										
B. mesentericus niger	I		0		0			6			0					1				
	II						8			5			96		94			97	80	71
	III	0		0		0			0			2		3		0				

However, it should be noted that since the experimental conditions were designed to duplicate those dealing with *B. mycoides*, optimum conditions for sporulation were quite probably lacking in some cases. For example, *B. subtilis* might have responded differently if a higher temperature or higher concentrations of peptone had been used. Probably an examination of the cultures after forty-eight or seventy-two hours of aeration would have revealed a decided effect of the amount of food supply on the percentage of spores produced.

The results obtained with *B. fusiformis* are of particular interest: the two experiments agree in showing that here spore production decreased in general as the amount of food supply in the medium decreased. This result is the opposite of that obtained with typical *B. mycoides* and similar to that obtained with the smooth variant of *B. mycoides*.

Recent observations indicate that *B. fusiformis*, like *B. mycoides* can undergo dissociation; whether the results of these two experiments hold true of one or all the types of *B. fusiformis* can be decided only by further investigation. The important point is that there are aerobic spore-formers which produce spores in quite different fashion from *B. mycoides* and that consequently no general explanation of the mechanism of spore formation seems possible at present.

DISCUSSION

The term "food supply" is used in this paper in a broad sense as including both organic and inorganic nutrients. The relative importance of these two types of constituents in the nutrition of *B. mycoides* can not at present be decided. Numerous attempts were made in this investigation to obtain a synthetic medium in which to grow *B. mycoides*, but they were all unsuccessful. Apparently, complex organic substances of a structure at present unknown are required. The possibility must not be neglected, however, that as the concentration of peptone is decreased the amount of elements such as zinc and copper and iron may become limiting factors. It is undoubtedly true that the supply of phosphorus in organic and inorganic form is a limiting factor. However, it is not the only influence concerned, since experiments have shown that the addition of phosphates in the form of NaH_2PO_4 to 0.25 per cent peptone (aerated) does not prevent the production of 80 per cent or more spores in twenty-four hours. The lack of material essential for the synthesis of protoplasm in the lower concentrations of peptone serves to initiate in the vegetative cells of *B. mycoides* internal changes resulting in spore formation. In this case the vegetative cell as such does not seem to be able to enter into a prolonged resting state; this seems to be possible with the smooth variant of *B. mycoides* and with other species of spore-formers such as *B. fusiformis.*

Both the amount of available food and the amount of metabolic products in the environment are of great importance in determining the extent of sporulation by *B. mycoides* under the conditions of these experiments. With relatively low concentrations of peptone the supply of one or more constituents essential to growth is exhausted before the accumulation of metabolic products retards growth and injures the vegetative cells. When the concentration of peptone is increased above 1 per cent (considering a volume of 70 cc. medium) the accumulation of metabolic products in the course of growth becomes the more important factor; if their concentration is sufficient, the vegetative cells are so altered that only a part of the population is capable of sporulation.

Measurements of the hydrion concentration of peptone cultures of *B. mycoides* aerated for twenty-four hours show that the alkalinity of the cultures parallels the amount of growth present, and increases with the amount of peptone in the medium. The maximum alkalinity attained was pH 8.1 (a twenty-four-hour, aerated, 4 per cent peptone culture), and while no systematic study of the effect of hydrion concentration has yet been made, it does not appear that the alkalinity of the medium is of much importance. When the aeration of cultures is extended beyond twenty-four hours, the alkalinity very probably has a much greater effect. Data on the hydrion concentrations of peptone cultures of the other spore-formers studied show that in these cases also the alkalinity in the course of twenty-four hours' aeration does not appear to have much effect on spore production.

In the case of organisms of the type of *B. fusiformis*, which produced an increased percentage of spores as the amount of peptone in the medium (aerated for twenty-four hours) increased, the accumulation of metabolic products seemed to be of much more importance than the food supply. With an increased food supply there was noted increased growth, which involves the production of a greater amount of metabolic products; correlated with this there was a greater spore production.

In view of the fact that the effect of the amount of peptone in the medium on spore production (after aeration for twenty-four hours) varied not only with different organisms, but apparently with the same organism according to its past cultural history, it is obvious that no general explanation—or even description—of the mechanism of spore production is possible at the present time. This apparent lack of a general mechanism as studied under the conditions selected in this investigation seems to offer a possibility of classifying the members of the Bacillaceae on a physiological basis. Any such study should be preceded by thorough investigations of dissociation phenomena of the various spore-formers concerned.

There is needed in the study of thermal resistance of spores a method for obtaining spores of a known and uniform state of resistance to heat or to chemicals. It is recognized by workers in this field that the same disinfectant tested on one lot of spores of a given organism may yield results quite different from those obtained with the same disinfectant under the same conditions on a second lot of spores. Whether the present technique in producing spores will yield material of a uniform degree of resistance (for a stable stock strain) remains to be seen.

In view of the facts that dissociation can alter the mechanism of spore formation of *B. mycoides* and that sporogenous variants of *B. mycoides* may be readily produced, the suggestion that the heat-resistant organisms responsible for food spoilage are, in the words of Morrison and Rettger (1930) "possibly super-resistant varieties of some common aerobic spore-forming organism because of the tendency to dissociation," is extremely interesting. They cite the work of Kelley (1926), who designated the causal agents of three types of spoilage in evaporated milk as atypical strains of *B. cereus, B. simplex* and *B. megatherium,* respectively. They themselves regard the aerobic spore-former which they isolated from cans of spoiled evaporated milk as a relatively stable variant of *B. vulgatus.* Whether a systematic study of the spores of a number of variants of a given spore-former will reveal consistent differences in their thermal resistance can only be decided by future investigations.

CONCLUSION

Studies on the production of spores by *Bacillus mycoides* Flügge showed that when this organism was grown in solutions of peptone of various concentrations and with different degrees of aeration the percentage of spores, as determined after twenty-four hours of incubation, increased as the concentration of peptone decreased and was consistently higher as the degree of aeration was increased.

The volume of growth increased with increase in the amount of peptone present but seemed to be unaffected by the degree of aeration above the minimum used in these experiments.

Dissociation may profoundly alter the mechanism of spore production; a culture of *B. mycoides* undergoing dissociation did not produce spores in any concentration of aerated peptone after twenty-four hours' incubation; also, it has been found that a variant of *B. mycoides* giving smooth, compact colonies on nutrient agar produced an increased percentage of spores as the amount of peptone in the medium was increased.

Studies of spore production in other aerobic forms gave variable results; some responded in a manner similar to *B. mycoides*; others either produced no spores at all under the conditions of the experiment or, as in the case of *B. fusiformis,* produced greater percentages of spores as the concentration of the peptone solutions was increased.

In the discussion and explanation of spore production by aerobes one must consider not only the kind of organism but also its physiological state.

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THE EFFECT OF "LOW VOLTAGE" X-RAYS ON THE ELECTROPHORETIC MIGRATION VELOCITY, VIABILITY AND pH OF ESCHERICHIA COLI SUSPENSIONS[1,2]

K. P. DOZOIS,[3] R. P. TITTSLER,[4] M. W. LISSE,[5] AND W. P. DAVEY[6]

The Pennsylvania State College, State College, Pennsylvania

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Bacteria under normal environmental conditions have a negative electrophoretic potential. Recently two of us, Lisse and Tittsler (1931), reported that the irradiation of *Escherichia coli* with ultraviolet or even Mazda bulb rays affected the electrophoretic migration velocity, viability, lysis and pH of an aqueous suspension. As a result of these studies we came to the conclusion that when these rays kill bacteria they first stimulate, a process accompanied by an increase in the electrophoretic migration velocity, and later destroy, a process accompanied by a decrease in this velocity. In brief, this work suggested the hypothesis that stimulation and injury are reversible processes in which the electrophoretic potential tends to return to normal, whereas death is an irreversible process in which there is no such return. The validity of this hypothesis may be questioned in view of the fact that Winslow, *et al.* (1923) reported "that heat-killed bacterial cells exhibit essentially the same curve of migration velocity as that of living cells."

According to the principles of photochemistry one is led to believe that certain effects, for example, those observed after

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[2] Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station as technical paper No. 542.

[3] Formerly assistant in Bacteriology.

[4] Formerly assistant Professor of Bacteriology, School of Agriculture.

[5] Professor of Biophysical Chemistry, School of Agriculture.

[6] Professor of Physical Chemistry, School of Chemistry and Physics.

irradiation with ultraviolet rays, are produced by a very specific range of wave lengths, and that rays of either longer or shorter wave lengths are less active. In this connection, however, it should be noted that Bovie (1916) showed that the Schumann rays are more reactive than the longer ultraviolet rays.

Warren (1928), in a review of "The Physiological Effects of Röentgen Radiations upon Normal Body Tissue" called attention to the injurious effects of these radiations upon body tissues, cell structures and life processes, and also noted that "it has been pointed out by many authors that these rays must be absorbed in order to be effective." Thus, it would seem that the injurious effects upon bacteria which have been noted by many workers must have been caused by absorbed radiations. This made us believe that if x-rays are absorbed by bacteria they may produce changes similar to those produced by ultraviolet irradiation.

The purpose of this investigation was to test the hypothesis advanced above by a direct determination of the effects of "low voltage" x-rays upon the electrophoretic migration velocity, viability, and pH of an aqueous suspension of *Esch. coli.*

So far as we are aware the literature contains no direct reference to the effect of x-rays upon the electrophoretic potential of bacteria. However, Fiorini and Zironi (1914) stated that the agglutinability of typhoid bacilli was not modified by x-rays. This would suggest no change in the electrophoretic potential according to the reports of two of us, Tittsler and Lisse (1928) and Lisse and Tittsler (1931).

The effects of x-rays upon the viability and physiological behavior of bacteria, and other microörganisms, have been studied extensively since Minck (1896) reported that typhoid bacilli were not injured by low voltage x-rays. The experimental methods and results of the early investigations have been reviewed thoroughly and adequately by Russ (1906), Klövekorn (1925) and Trillat (1927). The results of these studies differed greatly. Although some observers reported retarded growth, suppression of certain physiological characteristics such as pigment production, and even marked lethal action, there were many others who found that x-rays had little, if any, effect upon bacteria.

It would appear that x-rays either interfere with the growth and normal development of bacteria and yeasts, or produce a decided lethal effect according to many of the recent reports among which are those by Lacassagne (1928), Holweck (1929), Clark and Boruff (1929), Pauli and Sulger (1929), Holweck and Lacassagne (1930), Ellinger and Gruhn (1930), Wyckoff (1930a and 1930b) and others. However, Beckwith, *et al.* (1930) reported that the bacteria were not killed in their experiments.

Curie (1929) and Glocker (1931) have discussed the bactericidal action of x-rays from the quantum standpoint.

TECHNIQUE

Preparation of the suspension. The strain of *Esch. coli* used in the previous study of the effects of ultraviolet irradiation and which gave practically constant electrophoretic migration velocities during two years was used in this investigation. The cultures were grown on proteose-peptone agar slants of approximately pH 6.8 for twenty-four hours at 37°C. The twenty-four hour period of incubation was chosen because Shibley (1924) showed that the quantity of charge varied until the culture was about eighteen hours old, after which it remained rather constant. The growth was removed with a small volume of distilled water by a gentle rotary movement of the culture tube. This suspension was filtered through cotton, centrifuged at about 3,000 r.p.m. for one hour and the cells resuspended in distilled water. The washing was repeated twice more as advocated by Northrop and DeKruif (1922). A very concentrated suspension was prepared for irradiation as the structure of the x-ray apparatus did not permit the use of a large volume at one time. After irradiation the contents of six celluloid exposure cells (described below) were pooled and diluted to an arbitrary standard, using distilled water. The control suspensions were prepared by diluting a portion of the concentrated suspension in exactly the same ratio as the irradiated sample. Colorimetric measurements showed that the pH of the suspensions was approximately 6.2.

X-ray apparatus and technique. The source of the x-rays was a water cooled molybdenum target Coolidge tube mounted in the

General Electric x-ray diffraction apparatus, (Davey (1921 and 1922)). The tube was operated as a self-rectifying tube at 30 $K.V._{R.M.S.}$ The discharge current varied from 35 to 45 milliamperes. This current was read every minute so that the total radiation dose, expressed as the product of discharge current times time, could be expressed in milliampere-minutes at 30 $K.V._{R.M.S.}$ at 15.2 cm. distance (Mo target). No filters were used.

Exposure cells of approximately 50 by 10 by 3 mm. were made by forming a box of celluloid about the end of a piece of glass. These cells were very transparent to the radiation, and the shape gave as great and as uniform exposure to this radiation as was practicable. When filled with the aqueous suspension of bacteria, the cells were mounted at the lower openings of the x-ray apparatus and the protective door was closed as far as possible. Each cell, therefore, was irradiated by the direct rays from the Coolidge tube. The x-ray dosage (see table 2) ranged from 245 to 3,000 Ma-M at 30 $K.V._{R.M.S.}$ at 15.2 cm. distance (Mo target).

Measurements of electrophoretic migration velocity. The apparatus used was the capillary cell type described by Falk, *et al.* (1928). Since the migration velocity in this apparatus is dependent in part upon the bore and length of the capillary, preliminary tests were made upon a number of capillaries of uniform length. From these results several capillaries which gave almost uniform migration velocities were selected for use in this study. Having made such measurements on each capillary the later readings could be compared by simple proportion regardless of the individual capillary used. This precaution is quite essential because of the danger of breakage.

Measurements of migration velocities were made upon 10 bacterial cells (5 with each polarity of the electrical field). The capillary was then refilled and a second set of measurements was made. The two sets of measurements were averaged, provided their difference was not greater than 4 per cent. In case of greater differences further measurements were made. The results are expressed in terms of microns per second at 40 volts.

The capillaries and cell were cleaned thoroughly with dichromate-sulphuric cleaning solution and distilled water. Especial attention is called to the need for absolute cleanliness of all glassware and the use of only high grade distilled water.

Determination of viability and pH. In a portion of the study, ordinary plate counts and colorimetric measurements (Lamotte standards) were made upon both the irradiated and control suspensions.

TABLE 1

Electrophoretic migration velocities of repeated experiments using various capillaries

CAPILLARY	DATE	VELOCITY	AVERAGE	PERCENTAGE DEVIATION FROM AVERAGE
		μ/sec.		
1	January 28	27 17		−0 143
	January 29	26.99		−0 808
	January 30	27.12		−0 313
	February 26	27 57	27.21	+1 312
3	January 28	28.13		+1.699
	January 30	27.64		−0.072
	February 26	27.85		+0.687
	March 3	27.85		+0 687
	March 3	27 46		−0 723
	March 5	27 06	27 66	−2 133
5	January 28	26 92		+0 448
	January 30	26 68		−0 448
	January 31	26.80		0 000
	February 26	26 80	26.80	0 000
7	January 28	24 02		−1 395
	January 30	24.31		−0 246
	February 26	24.44		+0 328
	March 3	24 44		+0 328
	March 5	24 25		−0.451
	March 6	24.36		0.000
	March 7	24.36		0.000
	March 11	24 70		+1 395
	March 14	24.44		+0 328
	March 25	24 25	24.36	−0 451

EXPERIMENTAL RESULTS

Experiments, made to determine the experimental error in the hands of the operator, showed that almost identical readings could

TABLE 2

Summary of electrophoretic measurements

IRRADIATION DOSES (Ma-M AT 30 K.V.$_{R.M.S.}$ AT 15.2 CM. DISTANCE (Mo TARGET))	NUMBER OF TESTS	MIGRATION VELOCITY	
		Rayed	Control
		μ/sec.	μ/sec.
245–247	3	24.58	24.25
496–499	3	24.09	24.36
1,001–1,013	6	24.31	24.39
1,437	1	24.44	24.61
1,505–1,529	5	24 28	24 32
2,000–2,013	6	24 28	24.39
2,541–2,555	4	24.39	24.44
3,000–3,009	3	24.36	24 45

TABLE 3

Results of viability studies

IRRADIATION DOSES (Ma-M AT 30 K.V.$_{R M.S}$ AT 15.2 CM. DISTANCE (Mo TARGET))	NUMBER OF VIABLE CELLS PER CUBIC CENTIMETER	
	Rayed	Control
1,001	2,100,000	2,130,000
1,011	2,000,000	1,760,000
1,505	2,430,000	2,130,000
1,514	1,790,000	1,930,000
2,000	2,270,000	1,930,000
2,013	2,580,000	2,710,000
2,550	2,660,000	2,710,000
3,000	1,410,000	1,350,000
3,003	2,890,000	2,710,000

TABLE 4

Results of pH determinations

IRRADIATION DOSES (Ma-M AT 30 K.V.$_{R.M.S.}$ AT 15.2 CM. DISTANCE (Mo TARGET))	pH		
	Distilled water	Suspensions	
		Rayed	Control
1,001	6.2	6.2	6.2
1,514	6.2	6.2	6.2
1,514	6.4	6.4	6.4
2,000	6.2	6.2	6.2
2,013	6.2	6.2	6.2
2,555	6.6	6.8	6.6
3,000	6.0	6.2	6.2

be obtained for each individual capillary when suspensions of the culture were prepared upon different days as indicated in table 1. An inspection of the data shows that the readings with a single capillary did not vary more than 4 per cent, thus confirming the accuracy of the method as reported by Falk, *et al.* (1928), Chapman (1929) and in previous unpublished studies by ourselves.

The results of electrophoretic migration velocities (table 2), plate counts (table 3) and pH determinations (table 4), all show very clearly that the x-rays of the wave lengths studied did not alter the electrophoretic charge, viability or pH of *Esch. coli* suspended in distilled water of pH 6.2. Macroscopical examination of the irradiated suspensions revealed no evidence of lysis and plate counts (table 3) showed no lytic effect upon the living organisms. These results are quite different from those obtained with ultraviolet irradiation, Lisse and Tittsler (1931). In that case there was a marked change in electrophoretic velocity, a decided lethal effect, lysis and increase in the pH of the suspension.

The consistent electrophoretic velocities of controls (table 2) are in harmony with unpublished data obtained during two years and also with the recent report by Chapman (1929).

DISCUSSION

The changes in the electrophoretic migration velocity which accompanied the lethal effect of ultraviolet radiations suggested that if x-rays kill bacteria they should also produce changes in the migration velocity. On the contrary, if x-rays do not affect the electrophoretic migration velocity, no injurious or lethal effects should be expected in view of our hypothesis that injury and death are accompanied by changes in the migration velocity. The latter assumption is supported by the results of this investigation which show very clearly that neither migration velocity nor viability were affected. This does not exclude the possibility that these rays may produce changes in the migration velocity under experimental conditions which kill bacteria.

The fact that many investigators found injurious or lethal effects produced by x-rays while others, including ourselves, failed to detect any such effects indicates differences in experimental conditions. The literature shows that both biological and physical conditions have varied considerably from one investigation to another. The cultures used may have varied in sensitivity due to differences in both the nutritive medium and the period of incubation. The environmental conditions under which the bacteria were irradiated have also differed greatly since broth cultures, freshly inoculated agar plates and saline or water suspensions have been used. Undoubtedly the differences in quality and quantity of x-rays used have contributed greatly to the differences in the effects. However, in many instances little if any information was given concerning either the quality or intensity of rays emitted. Such variations render a comparison of the various investigations impossible. In view of the differences between the experimental conditions of the various investigations it is not surprising that different results have been obtained.

Using Mo rays at 34 to 38 $K.V._{R.M.S.}$, Wyckoff (1930b) found that a measureable proportion of *B. coli* was killed by doses of about one one-hundredth the amount which we found ineffective. Apparently there were only two important differences between his experimental conditions and ours. First, he irradiated organisms immediately after they were spread on the surface of agar, while we irradiated an aqueous suspension. Secondly, he used an x-ray tube having a Lindemann window which transmitted not only characteristic Mo-K rays but also very long and easily absorbed rays, while we used an x-ray tube made of the commercial type of glass. Even if the glass of our tube had permitted the passage of the extremely long rays they would have been absorbed by the first layers of the water suspension and therefore rendered practically ineffective. Wyckoff obtained very similar results with silver, copper, chromium and molybdenum targets in the x-ray tube, and the use of an Ag target gave almost identical results in the same time of exposure irrespective of whether he used a voltage of 21 $K.V._{R.M.S.}$ (giving Ag-L rays in addition to the continuous spectrum) or a voltage of 34 $K.V._{R.M.S.}$ (giving Ag-K rays in addition to the continuous spectrum). Therefore, we are tempted to assume that the Ag-K and Ag-L rays were no more effective than our Mo-K rays and that the lethal effect was due entirely to the long wave lengths of the continuous spectrum transmitted by the Lindemann window and absorbed by the bacteria on the agar surface.

There is also the possibility that the agar was influenced by the radiation in such a manner that the bacteria were affected by some secondary action which was not produced in our aqueous suspension. We are inclined to discount this possibility but do not believe that it can be entirely discarded. Further experiments are contemplated to test this point.

SUMMARY

1. The effects of "low voltage" x-rays (35 $\text{K.V.}_{\text{R.M.S.}}$) upon bacteria are discussed on the basis of a theory relating stimulation, injury and death to changes in the electrophoretic migration velocity.

2. During this and other investigations, the electrophoretic migration velocity of *Esch. coli* remained constant for over two years.

3. Using the total radiation from a Coolidge x-ray tube at 30 $\text{K.V.}_{\text{R.M.S.}}$ (without filters) no changes were detected in the electrophoretic migration velocity, viability or pH of an aqueous suspension of *Esch. coli* with exposures as great as 3000 milliampere-minutes at 15.2 cm. target-culture distance. This dose is about 100 times as great as that for which Wyckoff found a lethal effect. Possible causes for the difference in results have been discussed.

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ADSORPTION EXPERIMENTS WITH THE VIRUS OF VACCINIA

F. F. TANG

Department of Bacteriology, Medical College, National Central University, Woosung, Shanghai, China

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It is generally agreed that the loss of activity of the so called filtrable viruses resulting from filtration is due, at least in part, to the adsorption of the active agents on the walls of the filter pores rather than to the mechanical straining out of the virus particles. In our former filtration experiments with the viruses of vaccinia and herpetic encephalitis (Ward and Tang (1929); Tang (1930)) ,we have noticed that if the fresh virus-containing tissue were macerated with Pyrex fragments and suspended in hormone broth, the resultant filtrates were regularly more potent than the corresponding preparations made with the use of the ordinary glass powder or sand as the grinding material and isotonic saline solution as the dispersing menstruum. In an attempt to determine whether the superiority of hormone broth and Pyrex over the salt solution and common glass powder or sand was due to any difference in adsorption, this investigation was undertaken. Only the virus of vaccinia is included in the present study.

PREPARATIONS

Virus filtrates. Active cell-free filtrates of the virus were prepared by the method previously described by Ward (1929). It is of much interest to note that the virus may remain active in this condition for a considerable length of time. One of our preparations, filtrate 723, which was prepared on January 13, 1930, is still fully virulent up to the present—almost twenty months since it was prepared. It was put aside in the refrigerator and kept at 0° most of the time, occasionally 5° and rarely 10°C. The result of the titrations carried out at different periods is given in table 1.

Adsorbents. Aluminium hydroxide (electro-positive) and kaolin, kieselguhr or infusorial earth, animal charcoal, glass and sand (electro-negative) were chosen as adsorbents for the study. Aluminium hydroxide was prepared according to the technique of Willstaetter and Kraut (1923). A sufficient amount of ammonia ammonium sulphate solution was prepared by dissolving 300 grams of ammonium sulphate crystals in 420 cc. of 20 per cent ammonia solution. To 3250 cc. of this solution, previously warmed to 60°C., was added a hot solution of 250 grams of aluminium sulphate ($Al_2(SO_4)_3 \cdot 18H_2O$) in 500 cc. of water. During the process of precipitation and for a quarter of an hour afterwards the solution was vigorously stirred and the temperature kept at 60°C. The supernatant was decanted and the precipitate washed first with ammonium sulphate solution, then with

TABLE 1

DILUTION OF FILTRATE	TITRATION JANUARY 15, 1930	TITRATION SEPTEMBER 20, 1930	TITRATION APRIL 30, 1931	TITRATION AUGUST 30, 1931
1:10	+++	++++*	++++	+++
1:100	+++	++++	++++	+++
1:300	+++	+++	+++	+++
1:500	++	+++	+++	++
1:800	+++	+++	+++	++
1:1,000	++	+++	++	++
1:1,500	+	+++	++	++
1:3,000	++	++	++	++
1:6,000	++	++	++	++
1:12,000	++	++	+	+
1:24,000	++	++	+	++
1:48,000	++	+	+	++
1:120,000	±*	+	+	+
1:600,000		+	+	+
1:1,200,000		±	±	+

* In this and the following tables ++++ denotes a maximum reaction and ± a minute papule.

distilled water. In order to decompose completely the basic aluminium sulphate, an extra 40 cc. of 20 per cent ammonia water was added to the precipitate after the fourth decantation. The process of washing and decantation was continued for a dozen times or so until the washing was no longer clear. The precipitate was the aluminium hydroxide which when required was dried between filter papers.

Pyrex and ordinary glass powder was obtained by pulverizing scrupulously cleaned glass, and then passing the fragments through a No. 80 sieve. Sand, collected from the beach, was first washed with water then treated with 1 per cent HCl followed by 1 per cent NaOH. After neutralization it was washed again with distilled water then dried and sieved. Kieselguhr, kaolin, and animal charcoal were purchased.

Hormone broth. This was prepared by a modification of Huntoon's method. Four hundred grams of minced beef heart, 10 grams of peptone and 5 grams of salt were boiled for fifteen minutes in 1 liter of distilled water. The meat particles were then removed by straining through a perforated tin can, 24 cc. of N/1 NaOH added, and the broth autoclaved at 15 pounds for half an hour. It was then allowed to stand over night at the room temperature. Next morning the supernatant fluid was syphoned off, adjusted to pH 7.6 and autoclaved at 15 pounds for fifteen minutes.

Salt solution. It was prepared in the usual manner with 0.85 per cent NaCl, and the reaction was adjusted to pH 7.6.

Determination of the electrical charge. Although the opposite charge may not be the only thing that governs the process of adsorption of particles at a surface, nevertheless it is one of the most important factors concerned in the phenomenon. It is, therefore, desirable to determine what charge the vaccine virus carries under certain conditions before starting the adsorption experiments.

According to Olitsky and Boez (1927), the iso-electric point for the virus of foot and mouth disease is pH 8.0. By means of cataphoresis Douglas and Smith (1928) found that the vaccine virus carries a negative charge over a range of pH 5.5 to 8.2.

Following the simple "paper-creeping" technique recently devised by Bedson and Bland (1929), we have carried out a number of experiments with our filtrate, in which, we think, the virus exists in a purer state. Our result is in keeping with the finding of Douglas and Smith, and Bedson and Bland. The virus is negatively charged in a range from pH 6.4 to 8.2. The method we adopted was the following: strips of blotting-paper (Standard blotter (666), 0.5 cm. wide by 15 cm. long are cut out, and starting 0.5 cm. from one end, are marked in pencil with a centimeter scale up to 10 cm. They are then sterilized by dry heat. The virus filtrate when suitably diluted with the requisite buffer is placed in a small beaker to which a wire clamp has been fixed. A strip of the paper held at one end in the clamp is carefully lowered so that the free end dips into the virus suspension to the extent of 0.5 cm. The water is allowed to rise up to the 10 cm. mark, then the strip is withdrawn and the wetted portion cut into sample lengths, each being placed in an asigned place inside a Petri dish with a little of the buffer solution to prevent drying. All but the last three or four segments are tested in the rabbit for the presence of the virus. The first segment serves as a control, for the virus must be present there. The result of one of such experiments in which virus filtrate 759WA was diluted 1:10 in KH_2PO_4-NaOH and boric acid-KCl-NaOH mixtures is given in table 2.

TABLE 2

PAPER FRAGMENT	pH 6.4	pH 7.0	pH 7.6	pH 8.2
1	+++	++++	++++	++++
2	++	++	+++	+++
3	++	++	+	+
4	–	+	+	±
5	–	+	+	+
6	–	–	–	–

METHOD OF EXPERIMENTATION

Virus dilutions 1:100 were first prepared in hormone broth and salt solution separately. They were divided into control and test portions, each consisting of equal amounts in large test tubes. To the test portion, 1 per cent of the adsorbent to be investigated was added directly. This mixture together with the control was placed at room temperature for a certain length of time, usually one-half to one hour, with frequent shaking to facilitate adsorption. They were then centrifuged for a brief period so that the virus, if adsorbed, would be carried down by the particles of the adsorbent. The first 0.1 cc. of the supernatant and the last 0.1 cc. of the residuary fluid or sediment were diluted in distilled water or a pH 7.6 buffer solution and titrations carried out on rabbits by intradermal inoculations each consisting of 0.05 cc. of the requisite material. The animals were observed daily for one week but in tables 3 to 7 only the readings taken on the seventh day were recorded. Intradermal injections of 0.1 cc. of a 2 per cent suspension of all the adsorbents used in saline as well as in broth induced no reaction in the rabbit's skin except a very slight induration by kieselguhr and kaolin.

TABLE 3

Result of adsorption with aluminium hydroxide

Virus filtrate 723 was used. To 5 cc. of each test portion was added 0.05 gram of the aluminium hydroxide gel. Adsorbed for twenty, and centrifuged for fifteen minutes at 2000 r.p.m.

DILUTION OF FILTRATE	CONTROL (RABBIT 199)				ADSORPTION (RABBIT 192)			
	Saline		Broth		Saline		Broth	
	Supernatant	Residuary	Supernatant	Sediment	Supernatant	Sediment	Supernatant	Sediment
1:1,000	+++	++++	++++	++++	−	++++	++++	++++
1:10,000	++	+++	++++	+++	−	++++	++++	++++
1:50,000	++	++	+++	+++	−	+++	±	+++
1:100,000	+	+	++	++	−	++	+	++
1:300,000	−	±	++	+	−	+	−	++
1:1,200,000	−	−	+	+	−	+	−	−
1:4,800,000	−	−	−	−	−	−	−	−

RESULTS OBTAINED

We have carried out a large number of the experiments by the method described but for the economy of space we will present here only one of each kind made with a particular adsorbent. The results are given in tables 3 to 7.

TABLE 4

Result of adsorption with kaolin

To 1:100 dilution of virus filtrate 759 WB was added 1 per cent kaolin (Boluspulver, Merck). Adsorbed half an hour then centrifuged for fifteen minutes at 2000 r.p.m.

DILUTION	SALINE (RABBIT 205)				BROTH (RABBIT 206)			
	Control		Adsorption		Control		Adsorption	
	Supernatant	Residuary	Supernatant	Sediment	Supernatant	Residuary	Supernatant	Sediment
1:1,000	++	++	−	++	++	++	++	++
1:10,000	−	++	−	++	++	++	++	++
1:50,000	−	−	−	++	+	++	+	+
1:100,000	−	−	−	++	−	+	±	+
1:300,000	−	−	−	+	−	+	−	++
1:600,000	−	−	−	−	−	−	−	−

TABLE 5

Result of adsorption with animal charcoal

Virus filtrate 759 WB was used and diluted to 1:100. One per cent Merck animal charcoal was mixed. Left at the room temperature for half an hour then centrifuged for fifteen minutes at the usual rate. No control was made for this experiment, and rabbit 213 was inoculated.

DILUTION	SALT SOLUTION		HORMONE BROTH	
	Supernatant fluid	Sediment	Supernatant fluid	Sediment
1:500	−	++	++++	++++
1:5,000	−	++	++	+++
1:10,000	−	++	+	++
1:20,000	−	+	−	+
1:40,000	−	±	−	+
1:80,000	−	−	−	−

A study of the experimental data presented in tables 3, 4, 5, 6 and 7 reveals that the virus of vaccinia can easily be removed from the saline suspensions by the various adsorbents tested. There was only a slight amount of adsorption or practically none when the virus was suspended in hormone broth. In certain

TABLE 6

Result of adsorption with glass and sand

Ten per cent of the adsorbents was added to the dilutions of virus filtrate 759 WB. It was allowed to adsorb for one hour then centrifuged for ten minutes at 2000 r.p.m. Only the supernatant fluid was titrated.

DILUTION	SALINE (RABBIT 220)				BROTH (RABBIT 226)		
	Control	Pyrex glass	Common glass	Sand	Control	Pyrex glass	Common glass
1:100	++++	+++	++	+++	++++	+++	+++
1:500	++++	+++	+	++++	++++	+++	++
1:1,000	++++	++++	+	+++	++	+++	++
1:2,000	+++	+++	±	++++	+++	+++	++
1:4,000	++	++	±	++++	+++	++	+++
1:8,000	++	+	±	++	++	+	++
1:16,000	++	+	−	+	++	+	+
1:32,000	+	+	−	±	+	±	±
1:64,000	+	−	−	±	+	±	±
1:128,000	+	−	−	±	+	−	±

TABLE 7

Result of adsorption with kieselguhr

Virus filtrate 723 was diluted in distilled water as well as in broth, and 1 per cent kieselguhr was mixed with each. Two hours allowed for adsorption, then centrifuged.

DILUTION	CONTROL (VIRUS IN WATER, RABBIT 0844)		ADSORPTION (RABBIT 0824)			
			Distilled water		Hormone broth	
	Supernatant	Residuary	Supernatant	Sediment	Supernatant	Sediment
1:1,000	+++	+++	−	+++	++++	++++
1:10,000	+++	+++	−	+++	++	++++
1:50,000	+++	+++	−	++	++	+++
1:100,000	++	++	−	++	+	++
1:300,000	++	+	−	+	±	++
1:1,200,000	+	+	−	+	−	+
1:4,800,000	+	+	−	+	−	+
1:60,000,000	−	±	−	−	−	−

experiments distilled water titrated to pH 7.6 has been used instead of salt solution and it was found that it behaved like salt solution.

Common glass is definitely more adsorptive than the Pyrex but it is interesting that sand is almost non-adsorptive even in the saline suspension of the virus as shown in table 6.

EFFECT OF SALT SOLUTION UPON THE VIABILITY OF THE VIRUS

In the course of our experimentation, we have noticed repeatedly that the virus when diluted in salt solution often gave a lower reading upon titration than the corresponding preparations made in hormone broth. From what we have found about the herpes virus (Zinsser and Tang (1926)), we thought that the salt solution may possibly exercise the same deteriorating effect on the virus and might produce the difference in titer when comparing with the hormone broth suspensions. In order to determine whether this supposition might not be a fact, we have conducted experiments as follows: Parallel dilutions of the virus are prepared in salt solution and hormone broth of the same reaction (pH 7.6). As it was Gordon's preference to use distilled water as a diluent for the virus (1925), we have included it in the test to see if it is any better. Each series is subdivided into several sets in Wassermann tubes. One set is titrated as soon as it is ready and the rest placed in an incubator at 36°C. and titrations carried out at varying lengths of time. The result of one such experiment made with virus filtrate 759WB is illustrated in table 8. Rabbits 221, 222, 223, 224 were inoculated.

TABLE 8

DILUTION	SALT SOLUTION				DISTILLED WATER				HORMONE BROTH			
	2 hours 26°C.	3 hours 36°C	6 hours 36°C.	9 hours 36°C.	2 hours 26°C.	3 hours 36°C.	6 hours 36°C.	9 hours 36°C.	2 hours 26°C.	3 hours 36°C.	6 hours 36°C	9 hours 36°C.
1·10	++++	+	++++	+	+++	++	++	++	++++	++++	+++	++
1:100	+++	+	+++	++	+++	+++	+++	+++	++++	+++	+++	++
1:500	++	++	+++	+++	++	++	++	++	+++	+++	++	+
1.1,000	+	+++	++	+++	+	+++	++	++	+	+++	++	+
1.2,000	+++	+++	++	+++	+	+++	++	++	++	++	++	++
1:4,000	++	+	+	+	+	++	++	++	++	++	+++	+++
1:8,000	++	+	+	−	++	+	+	−	+++	+	++	++
1·16,000	++	+	−	−	++	±	+	−	+++	++	++	++
1.32,000	++	±	−	−	+	−	−	±	++	++	++	+++
1:64,000	−	−	−	−	+	−	−	−	++	++	+	++
1:128,000	−	−	−	−	−	−	−	−	+	+	+	++
1:256,000	−	−	−	−	−	−	−	−	+	+	+	+

This and similar experiments show that the virus rapidly loses its virulence in salt solution or distilled water. There was a distinct diminution of infectiousness even when kept at room temperature for two hours. When incubated at 36°C. for nine hours the infectivity had deteriorated more than 50 per cent. On the other hand there was not the slightest change in the titer throughout the experiment when the virus was dispersed in hormone broth.

DISCUSSION

The experiments reported in this paper have demonstrated beyond any doubt that the virus of vaccinia can be easily adsorbed from salt solution or water but not from hormone broth by the various adsorbents tested. The experiments have been consistent and clear cut.

The nature of the substance in the broth which inhibits adsorption is not yet clear but perhaps owing to the presence of the so called "hormones" or X substances, the broth may possess greater affinity for the virus molecules, so that a firm union is established as soon as the two are brought together and as a consequence the encroachment of a second adsorbent is interrupted or prevented. This phenomenon may offer an adequate explanation of the successful filtration of several viruses such as the vaccine virus, neurovaccine, herpes and rabies (unpublished experiment of the writer) in hormone broth but not in salt solution as we have previously communicated (Ward and Tang (1929); Tang (1930)). When suspended in broth, the virus is kept from being adsorbed by the filter and thus its passage through the pores is rendered free. When salt solution is used as the menstruum the condition is just the reverse. Here, much of the virus is removed by the filter candle and little or none at all is allowed to pass through, consequently the filtrate is only slightly active or completely inactive.

Several years ago, while working on the neutralization power of the herpes immune serum, we (Zinsser and Tang (1926)) showed that the herpes virus rapidly loses its virulence in saline. The same has been found to be true with the vaccine virus in this study. This fact serves to illustrate further the inferiority of salt solution when used as a medium to suspend the virus before filtration, for any virus which escaped the adsorption process and passed through the filter would be further subjected to the action of the salt solution which may render it innert within a short while. Whether the deterioration of the virus in salt solution is due to oxidation as we (Zinsser and Tang (1929)) have shown in case of the herpes virus, has not yet been determined.

Pyrex is definitely less adsorptive than the ordinary glass, but sand, when compared weight by weight, is practically non-adsorptive. Its inferiority as a grinding substance probably lies, therefore, in its not possessing as sharp edges as the Pyrex fragments, as Ward (1929) has conjectured.

From a theoretical point of view, if the phenomenon of adsorption is due to the attraction of opposite charges, then we have no explanation for the adsorption of the virus by kaolin, kieselguhr, glass and animal charcoal, for they, as well as the virus itself, are all similarly negatively charged.

Certain preparations of animal charcoal may inactivate the virus even using only 0.2 per cent of the chemical. This is apparently due to the impurities present rather than to the chemical itself.

SUMMARY

A series of adsorption experiments have been conducted with various adsorbents on the virus of vaccinia suspended in salt solution as well as in hormone broth. It has been demonstrated that the virus can easily be adsorbed from the saline suspension but not from the hormone broth.

Pyrex is definitely less adsorptive than the ordinary glass fragments but sand is practically non-adsorptive so that its inferiority when served as a grinding material cannot be attributed to adsorption.

The writer wishes to express his thanks to Dr. Dunscombe of the Shanghai Municipal Health Department and Dr. Tsen of the Hygienic Laboratory of the Municipality for Greater Shang-

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AN APPLICATION OF THE AUTOCATALYTIC GROWTH CURVE TO MICROBIAL METABOLISM[1]

H. C. PULLEY AND J. DUDLEY GREAVES[2]
Utah Agricultural Experiment Station, Logan, Utah

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Growth does not take place at a constant rate in living organisms. In bacterial cultures it is initially exceptionally slow, then increasingly rapid, and finally exceptionally slow. This is most conspicuous if the initial inoculum into fresh media is very small. We have found that the rate of accumulation of microbial metabolic products likewise is not constant but begins slowly, increases rapidly, and again slows down.

The work reported concerns the accumulation of nitrates by soil bacteria and of carbon dioxide by yeasts. During the earlier part of the incubation period the accumulation of nitrates was meagre. Then followed a period of rapid increase and following this a very slow or negative accumulation. In some cases as much as 70 mgm. of nitric nitrogen had accumulated between the twelfth and the sixteenth days, whereas only 20 mgm. had accumulated up to the twelfth day. The same relationship holds for carbon dioxide production by yeasts, but the changes in rate are less extreme.

If the concentration of nitrates or carbon dioxide be plotted against time it may be observed that the increase is regular (not constant) and that the plotted points conform closely to a smoothly drawn S-shaped curve. This is typical of growth curves to which considerable study has been devoted. An inclusive list of references to the literature on the subject of time growth relations up to 1926 has been published by the Missouri Agricultural Experiment Station (Brody, Hogan et al. (1926)). Both weight and linear increase have been used to indicate growth.

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[1] Contribution from Department of Bacteriology, Utah Agricultural Experiment Station.

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This study was planned by Dr. J. E. Greaves and carried out under his supervision. Valuable assistance was also given by Dr. Willard Gardner.

[2] Assistant Bacteriologist and graduate student, respectively.

Robertson (1923) has applied the differential equation which expresses the speed of a monomolecular autocatalyzed chemical reaction to the growth of plants and animals. In this paper that equation is applied to our data on nitrate and carbon dioxide accumulation.

The equation implies that the catalyst is produced by the reaction in exactly the same proportion as the end-product y accumulates. It then becomes one of the reacting substances. By letting a represent the original concentration of the substrate, y the amount used by the reaction (the product) and also, by our assumption, the catalyst, and k_1 the proportionality constant, the rate of reaction may be expressed as

$$\frac{dy}{dt} = k_1 y(a - y) \tag{1}$$

However, the amount of nutrient substrate a is not generally the limiting factor of growth, but the reaction must be considered reversible, and an equilibrium is reached which is expressed by subtracting the reverse from the forward reaction.

$$\frac{dy}{dt} = k_1 y(a - y) - k_2 y^2 \tag{2}$$

It is understood that the catalyst is active in both directions. A second constant k_2 must be introduced for the reverse reaction. By a little manipulation and substituting A for $\frac{k_1\,a}{k_1 - k_2}$,

$$\frac{dy}{dt} = y\,\frac{k_1\,a}{A}\,(A - y) \tag{3}$$

which is, since $\frac{k_1\,a}{A}$ is a constant, identical in form with (1). But A is a fractional part of a and defines the limit of growth or maximum amount of end-product due to equilibrium between the forward and reverse reactions rather than the total amount of nutrient substrate originally present.

Integrating (3) and simultaneously substituting K for $\frac{k_1\, a}{2.3\, A}$ (changing to Napierian logarithms), we obtain

$$\log \frac{y}{A - y} = Kt + C \tag{4}$$

We wish to evaluate C as $-Kt_1$ where t_1 is the time at which the reaction is half completed, that is, $y = \frac{A}{2}$.

$$\log \frac{y}{A - y} = K(t - t_1) \tag{5}$$

At the point of maximum velocity the acceleration, expressed by the second derivative, is 0, from which we calculate $y = \frac{A}{2}$. The curve is symmetrical with its center at $y = \frac{A}{2}$, $t = t_1$.

When solved explicitly for y

$$y = \frac{A \cdot 10^{K(t - t_1)}}{1 + 10^{k(t - t_1)}} \tag{6}$$

EVALUATION OF CONSTANTS

In applying this equation to experimental data the three constants, A, K, and t_1, must be properly evaluated. They were calculated from a modified form of equation ~~(3)~~ in which $b = Kt_1$.

$$\log \frac{y}{A - y} = Kt - b \tag{7}$$

The constant A, representing the amount of end-product, in most cases was evaluated by inspection after plotting the data. It is approximately an arithmetic average of the several determinations made after the maximum had been reached. In some cases

A was evaluated by a method Robertson uses (1923, pp. 63–64), substituting in four equations four values of t and y such that when the first is subtracted from the second and the third from the fourth, the two differences are equal. This means the four points must be chosen in two pairs separated by equal time intervals. A can then be calculated. It is desirable to choose points well distributed along the curve. However, the choice is quite arbitrary and the method so overweights those points chosen that a more satisfactory A can be chosen by inspection. The constants K and b were evaluated by the method of least squares.

NITRIFICATION

The data plotted in the nitrification curves which follow (figs. 1 to 16, inclusive) were obtained by inoculating soil (1 gram) into Fred's nitrite medium (200 cc.) and incubating for five weeks. The nitrates present in 5 cc. of solution were determined daily for twenty-one days by the phenoldisulphonic-acid method and after that on the twenty-ninth and thirty-sixth days.

Both productive and alkali soils were used to inoculate. The productive soil was also artificially made alkaline by the addition of salts. Each soil was used both before and after being leached with water (Greaves and Pulley (1931)). In table 1 are listed the various soils used, together with a summary of the equation constants for nitrification.

The curves plotted conform so uniformly to the data obtained experimentally that there can be no doubt that the progress of metabolism indicated by nitrate formation is autocatalytic, conforming to the general mathematical expressions given above.[3]

DISCUSSION OF EQUATION CONSTANTS

The velocity constant K is influenced by the type of soil. This may be due in part to the modifying effect the soil has on the type of microflora present and in part to the stimulating or the toxic effects of the salts added with the soil. A salt which stimulates nitrification increases K. One which depresses lowers its value.

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[3] A similar study is being conducted at the Utah Station on the time relationship of NH_3 accumulation by soil organisms.

Sodium carbonate is highly stimulating if present in small quantities but becomes toxic in slightly larger amounts. Media inoculated with soil (1 gram in 200 cc.) carrying 1 per cent Na_2CO_3 and 1 per cent NaCl have an exceptionally high *K*, 1.05, which by leaching is lowered to 0.37. That inoculated with soil carrying 2 per cent Na_2CO_3 is toxic; here *K* is only 0.12. But the same soil leached has a *K* of 0.51. The native alkali soil of Benson

TABLE 1

Soils used to inoculate nitrite media for nitrification, also calculated equation constants

SOIL NUMBERS	DESCRIPTION OF SOIL	EQUATION CONSTANTS		
		K	t_1 (days)	*A* (milligrams nitric nitrogen)
53–54	Native soil + 1 per cent each NaCl, Na_2CO_3	1 05	21.0	88
45–46	Native soil + 1 per cent each NaCl, Na_2SO_4	0 62	19.9	93
35–36	Native soil + 2 per cent Na_2CO_3, leached	0 51	13.1	95
21–22	Native soil + 2 per cent Na_2SO_4	0 41	17.2	94
69–70	Native soil + $\frac{2}{3}$ per cent each, NaCl, Na_2SO_4, Na_2CO_3	0 38	14.1	92
79–80	Corinne soil, alkali	0 37	21.5	90
81–82	Corinne soil, alkali leached	0 37	16.8	92
55–56	Native soil + 1 per cent each, NaCl, Na_2CO_3, leached	0 37	13.4	94
71–72	Native soil + $\frac{2}{3}$ per cent each, NaCl, Na_2SO_4, Na_2CO_3, leached	0 37	15 5	95
89–90	Richland acres soil, alkali	0.35	15.7	98
61–62	Native soil + 1 per cent each, Na_2SO_4, Na_2CO_3	0 35	15.9	94
93–94	Richland acres soil, alkali, leached	0 32	15.3	94
11–12	Native soil + 2 per cent NaCl, leached	0.31	16.4	96
23–24	Native soil + 2 per cent Na_2SO_4, leached	0 30	16.8	98
47–48	Native soil + 1 per cent each, NaCl, Na_2SO_4, leached	0 26	16 9	90
1–2	Native soil	0.26	16.7	105
3–4	Native soil, leached	0 25	16 7	104
63–64	Native soil + 1 per cent each Na_2SO_4, Na_2CO_3, leached	0 24	15.0	94
101–102	Benson soil, alkali	0 22	28 2	88
105–106	Benson soil, leached	0 16	26.0	92
33–34	Native soil + 2 per cent Na_2CO_3	0 12	39.9	95

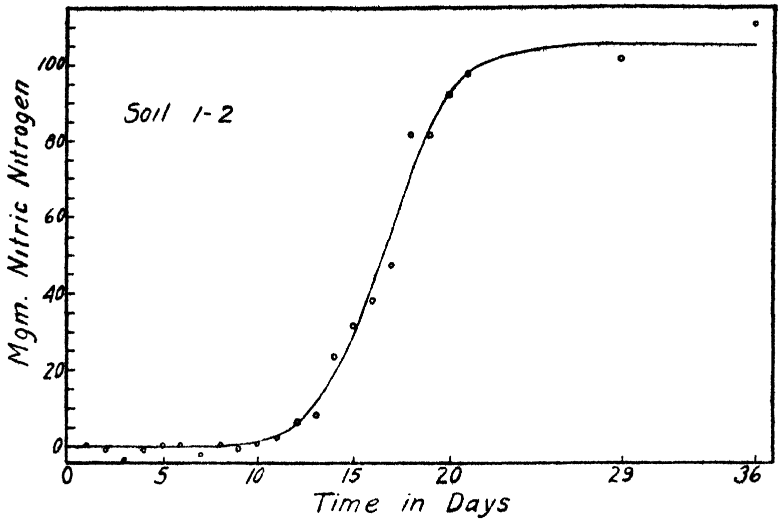

FIG. 1

FIGS. 1–16, inclusive. Time rate of nitrate accumulation in nitrite media inoculated with 1 gram of various soils. Soil numbers refer to table 1. The disconnected points represent experimental observations. The smooth curve is calculated from formula 3.

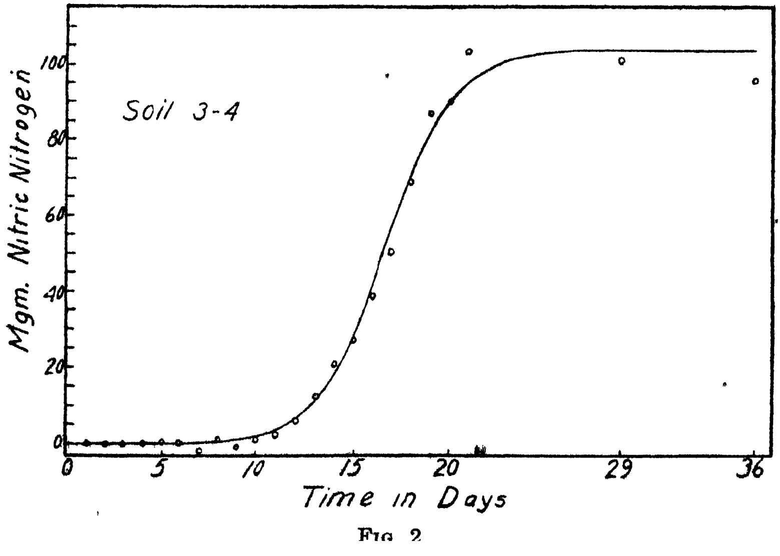

FIG. 2

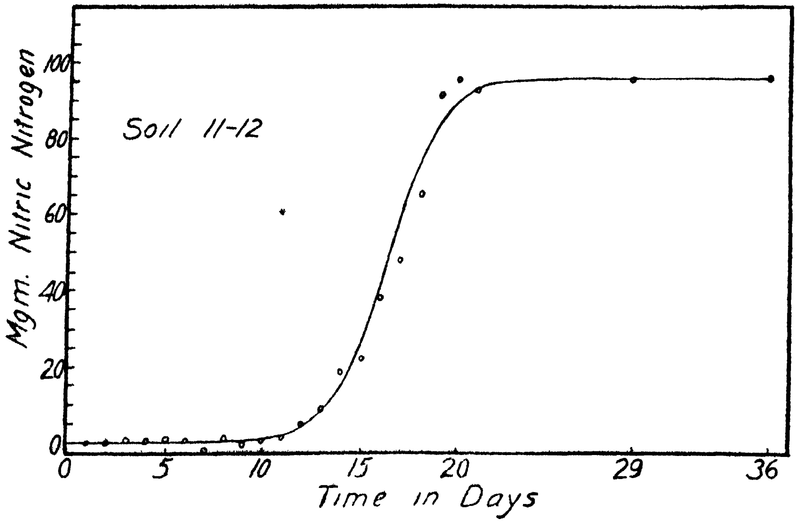

FIG. 3

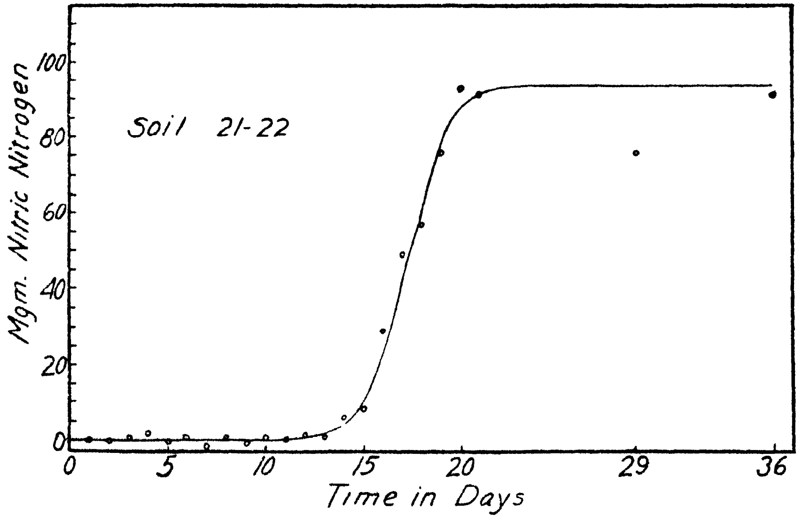

FIG. 4

gives a low *K* most comparable to the toxic carbonate soil, but in this case it is not increased by leaching. Sodium carbonate in

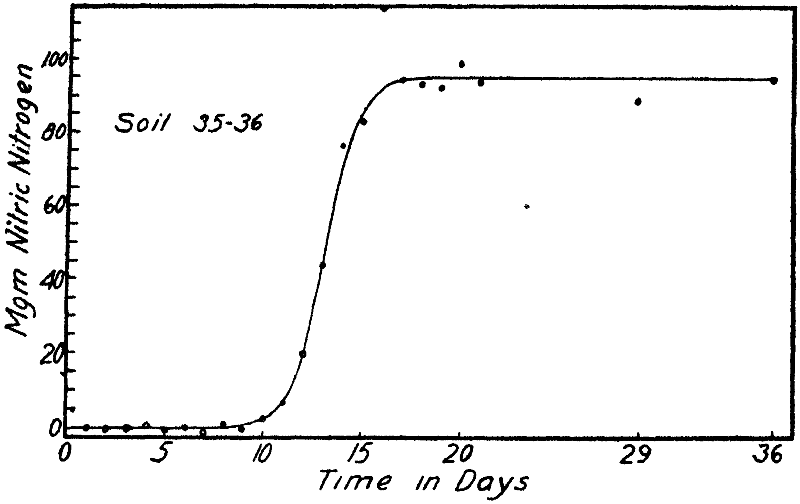

FIG. 5

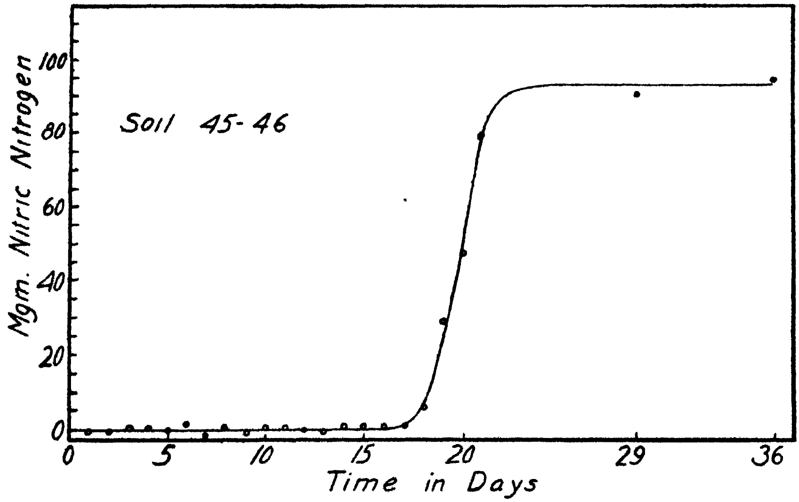

FIG. 6

combination with the sulfate and with the sulfate and chloride is less stimulating.

Nitrifiers are less sensitive to sulfate than to carbonate. The

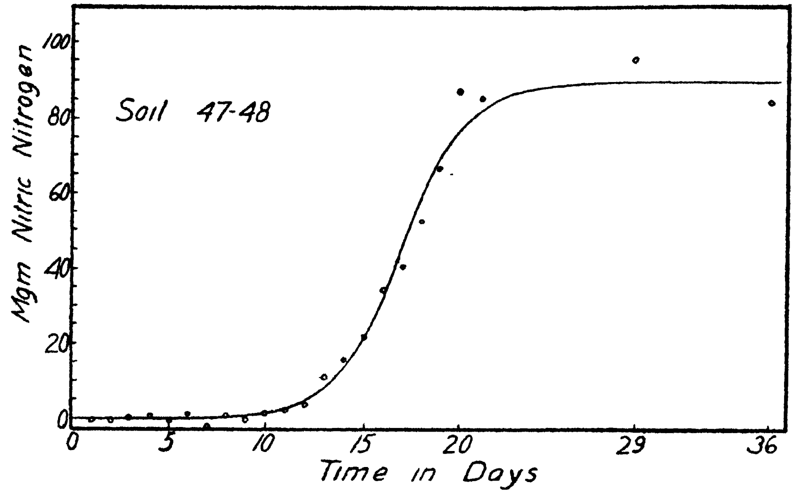

FIG. 7

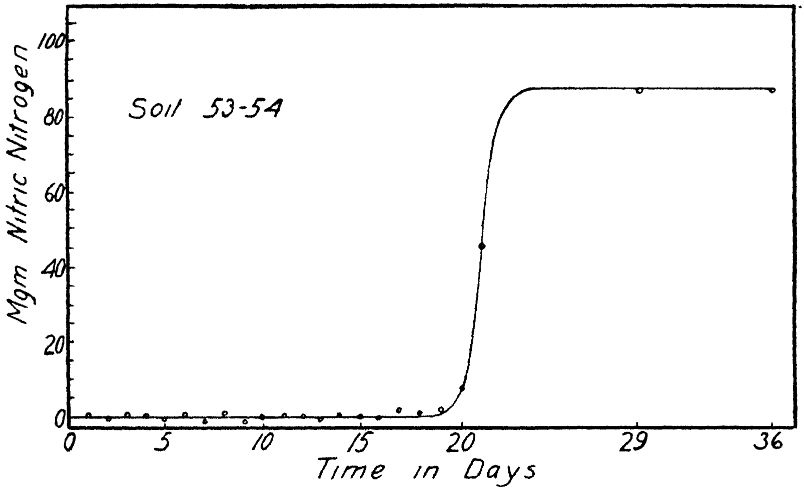

FIG. 8

soil carrying 2 per cent Na_2SO_4 gives $K = 0.41$. The same leached is only 0.30. In smaller amounts stimulation is less pronounced. Sodium chloride also stimulates in the concentrations used but more so in non-leached soils. The 2 per cent NaCl

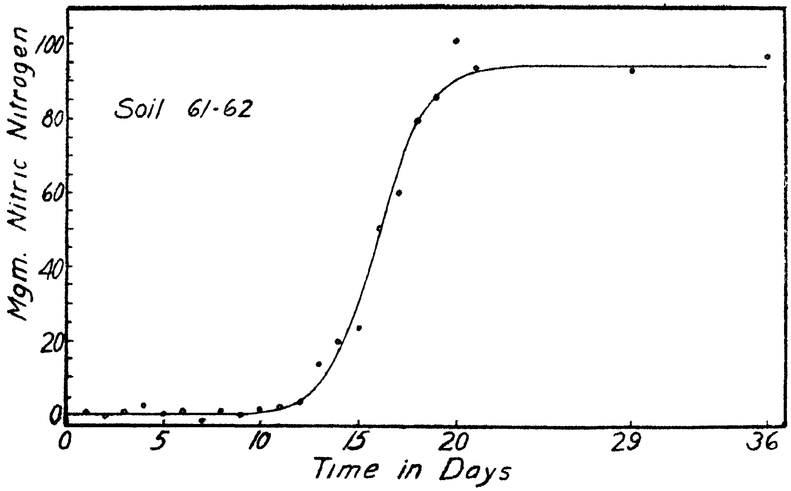

FIG. 9

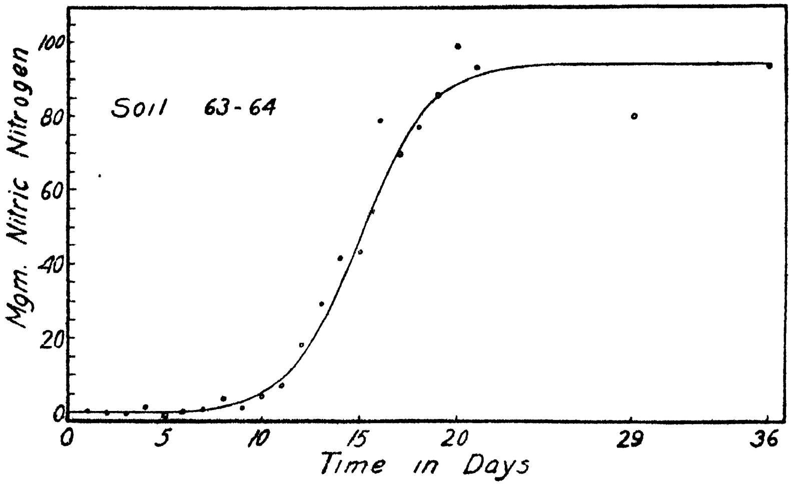

FIG. 10

non-leached soil was lost from the series. The NaCl-Na_2SO_4 combination is stimulating, K being 0.62.

The time t_1, in which the "nitrate cycle" is half completed, is not without significance. With the 2 per cent Na_2CO_3 soil it is

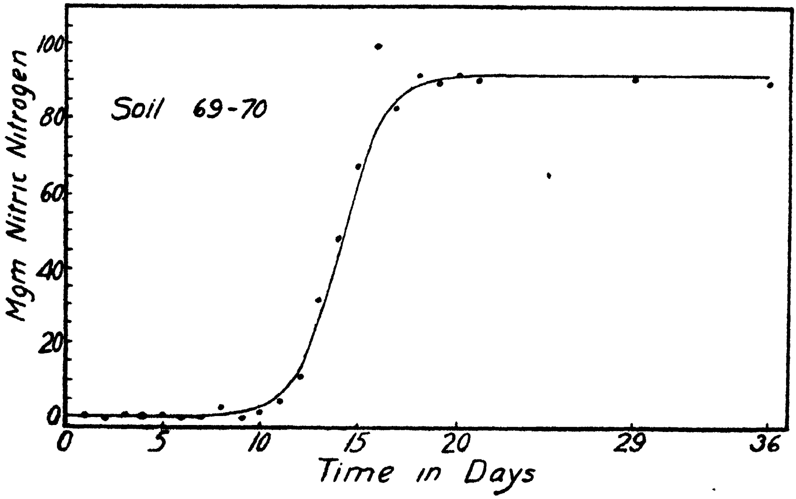

FIG. 11

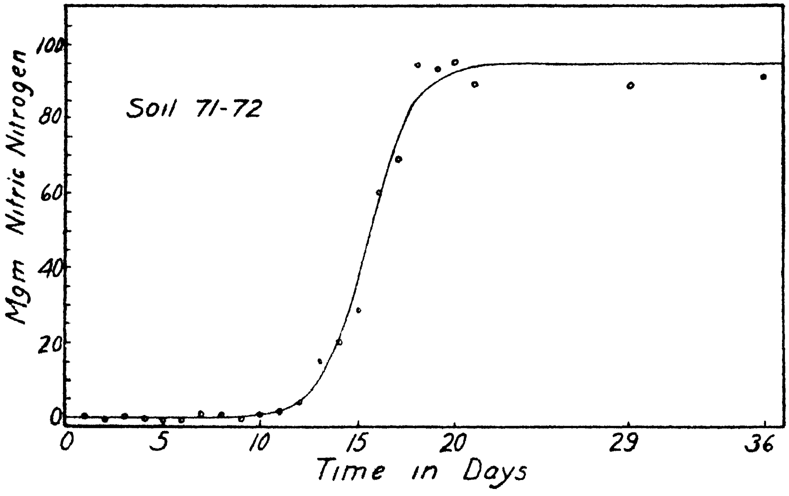

FIG. 12

39.9 days. After leaching this soil, t_1 becomes 13.1 days. The alkali Benson soil is also "slow." The most usual effect of leaching is to shorten t_1. A long lag period (high t_1) is accompanied by a low velocity constant K.

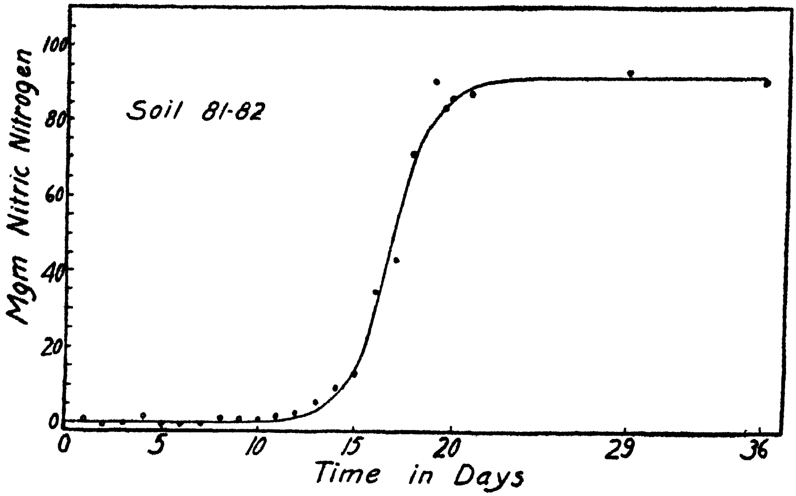

FIG. 13

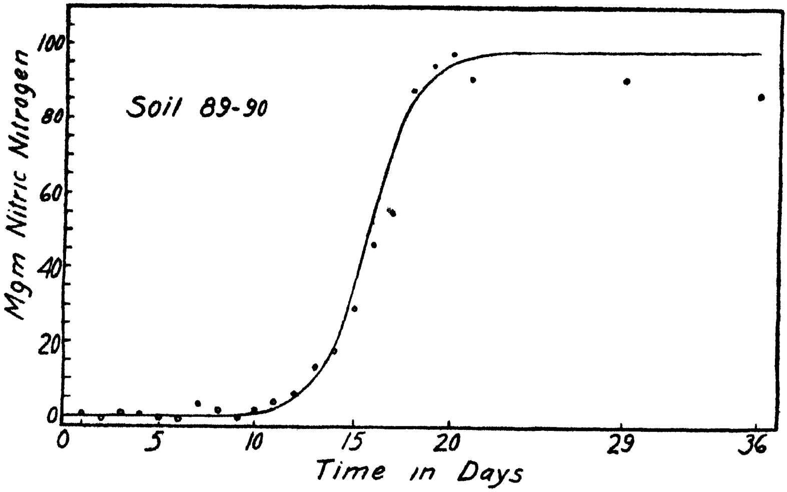

FIG. 14

Leaching is without significant influence on A, the ultimate amount of nitrates formed. This is maximum in native productive soil, both leached and non-leached, but is minimum in 1 case in a toxic alkali soil and in another case in a highly stimulated

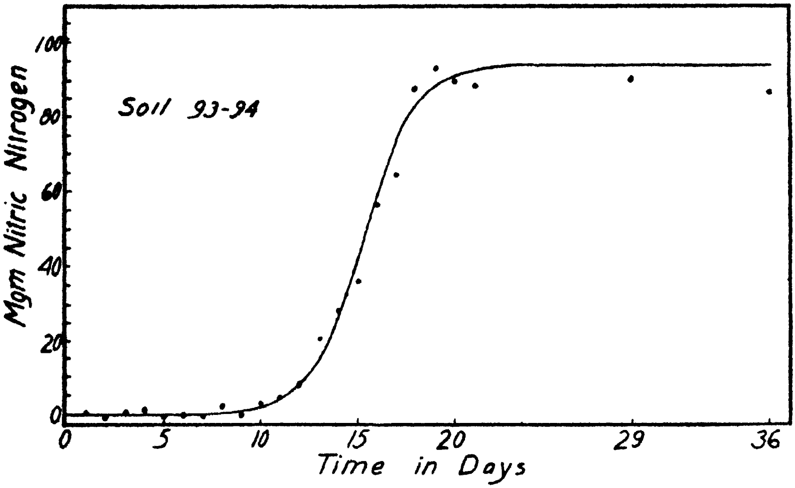

FIG. 15

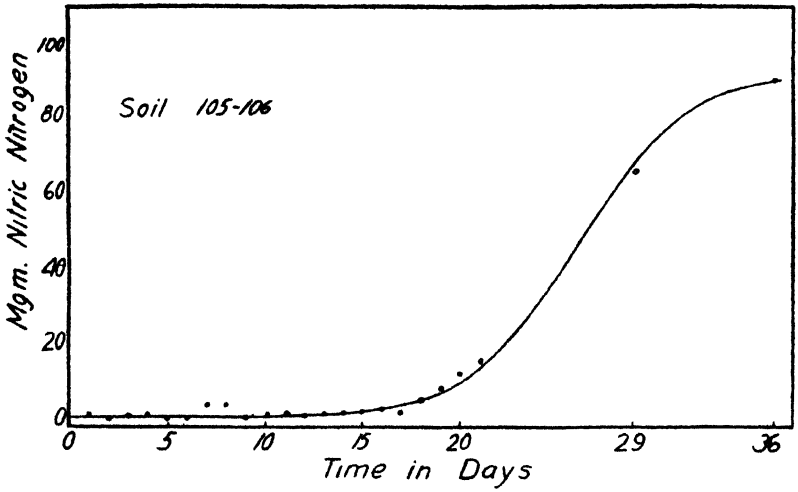

FIG. 16

soil. The constant A is not highly variable but t_1 is. Hence, a better insight into nitrifying powers can be obtained, at least in liquid media, by using an incubation period of fifteen to eighteen days rather than longer. At this time, t_1, the respective curves are most widely divergent, tending in the progress of time to converge to an approximately uniform maximum, A.

YEAST METABOLISM

In a study on the influence of iodine on the growth and metabolism of yeasts (Greaves, Zobell and Greaves (1928)) considerable data were collected on the rate of carbon dioxide evolution. Both commercial yeasts and pure cultures of *Saccharomyces cerevisiae* were cultured. The initial inoculation varied in different cases from 1.5 to 480,000 cells per cubic centimeter of solution. During short incubation periods the carbon dioxide was measured every two hours after an initial lapse of twenty-four hours. Other tests were run for seven, nine, fourteen, and thirty-one days, the carbon dioxide being measured either daily or every two days. Mayer's culture fluid was used as the medium, modified in various cases by the addition of different sugars and of NaI, KI or I_2 in amounts from 0 to 8000 p.p.m.

Counts of the yeast were made by diluting to the required extent and then placing a drop on the disk of a hemocytometer. Ten groups of 25 squares were counted. In the absence of agreement among duplicates this was repeated. The reported results are the average of a number of determinations and represent the number of yeasts found in one cubic millimeter of the cultural solution at the specific time. One of the greatest obstacles encountered in the work was the obtaining of representative samples as the yeasts tend to adhere.

The data obtained furnish an excellent opportunity to apply the autocatalytic growth formula to yeast metabolism. The determinations were made with reasonable accuracy, and check samples show that they are consistent. The data used in our curve study were obtained from seven series of flasks. The various series differed in the iodine treatment. Each consisted of from 6 to 12 different concentrations of the iodine, in duplicate or triplicate. The data, therefore, include more than 150 culture flasks. The curves which follow are typical of those studied. The equation constants for the theoretical curves are given in table 2.

TABLE 2

Data to accompany figures 17 to 24

The amount of initial inoculation, the incubation period and the media are listed, also the equation parameters for the curves.

FIGURE NUMBER		INCUBATION PERIOD	INITIAL INOCULATION	MEDIA	IODINE TREATMENT	EQUATION CONSTANTS					
								Days		Milligram CO_2	
						K	K'	t_1	t'_1	A	A'
		days									
17	I	1.54	Commercial yeast, 480,000 cells per cubic centimeter	Mayer's with commercial beet sugar	No NaI	1 66		1.65		623	
	II	1 54			1 p p.m. NaI	1 63		1.76		900	
	III	1.54			10 p p.m. NaI	1 87		1 66		825	
	IV	1.54			100 p p.m. NaI	1 85		1.55		627	
18	I	1 5	Commercial yeast, 480,000 cells per cubic centimeter	Mayer's with commercial beet sugar	1 No I_2	2 45		1 09		170	
	II	1.5			1 p p m. I_2	1.73		1.14		200	
	III	1.5			10 p.p m. I_2	2 02		1 17		170	
	IV	1.5			100 p.p.m. I_2	2 26		1.31		130	
19		9	Commercial yeast, 480,000 cells per cubic centimeter	Mayer's with commercial beet sugar	No KI	1 78	0 31	1.89	538	530	280
20		9			100 p.p.m. KI	1 73	0 31	1 86	555	500	330
21	I	7	*Saccharomyces cerevisiae*, 100 cells per cubic centimeter	Mayer's with sucrose	100 p.p m KI	0 52		5 17		550	
	II	7			1,000 p.p.m. KI	0 63		4 73		530	
22	III	7	*Saccharomyces cerevisiae*, 50 cells per cubic centimeter	Mayer's + dextrose	10 p.p.m. KI	0.25		10 3		260	
	IV	14		Mayer's + purified sucrose	1,000 p.p.m. KI	0 22		6 83		280	
22	I	31	*Saccharomyces cerevisiae*, 150 cells per flask of 100 cc.	Mayer's from purified chemicals with especially purified sucrose	No KI	0.10		21 0		245	
	II	31			10 p.p.m. KI	0 09		24.7		500	
23		31			100 p.p.m. KI	0 11		23 4		650	
24		31			1,000 p.p.m. KI	0 15		17 9		536	

In the first experiments conducted, each culture flask received an initial inoculation of 480,000 cells per cubic centimeter of culture solution. With this inoculation rapid growth took place and the carbon dioxide evolved was measured every two hours from the twenty-fourth and thirty-sixth hours. From the determinations curves were calculated by the method already described to fit the experimental observations. Some of these curves are shown in figures 17 and 18 and their equation constants in table 2. K is large for these curves and t_1 is low.

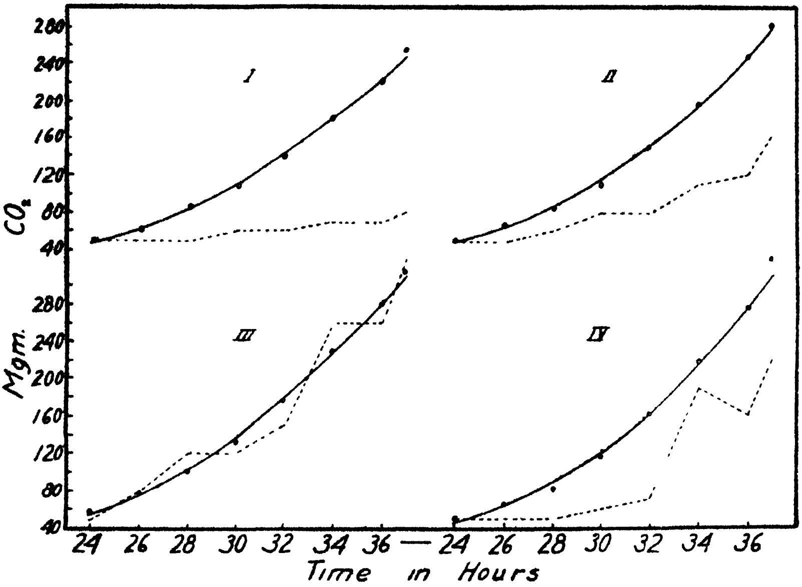

FIG. 17

FIGS. 17–24, inclusive. Time rate of CO_2 evolution by commercial and pure yeast cultures in Mayer's culture fluid varying the carbohydrate and the iodine content of the media and the amount of the initial inoculation. The dots represent experimental observations and the smooth curve the theoretical values. The ordinate scale represents also in hundreds of thousands the counts made of the number of yeast cells per cubic centimeter of solution, which is indicated by the dotted line.

The variations in treatment and inoculation, together with the equation constants for each of the smooth curves, are given in table 2.

Another set of flasks were similarly inoculated and observed, but the incubation and carbon dioxide measurements were continued for nine days. Curves from this set are shown in figures 19 and 20. Up to the thirty-seventh hour they closely correspond to those in figure 17. The equation constants are also similar. However, the later observations during the longer period of incubation digress considerably from the theoretical curve. But this digression again fits an autocatalytic curve calculated from the same general equation (Equation 3) with a different set of constants. The value of y is the sum of two others obtained for the two curves separately.

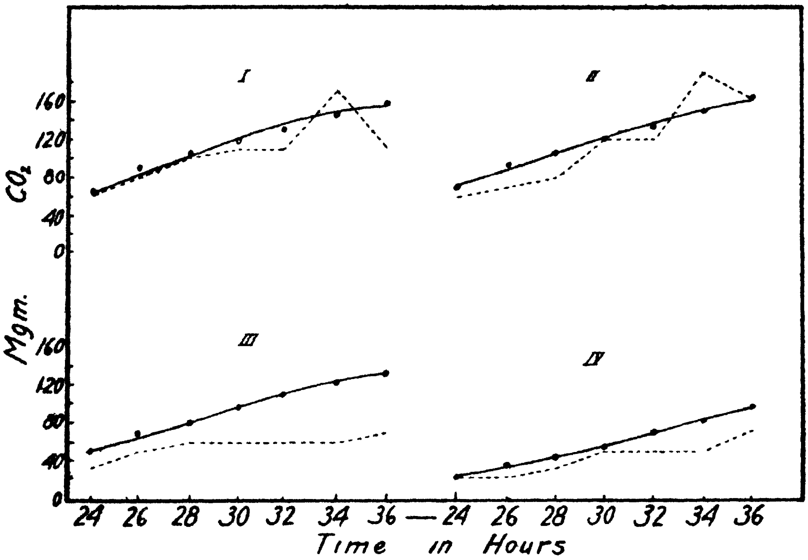

FIG. 18

$$y = y_1 + y_2 \qquad \begin{aligned} y_1 &= \frac{A \cdot 10^{K(t - t_1)}}{1 + 10^{K(t - t_1)}} \\ y_2 &= \frac{A' \cdot 10^{K'(t' - t_1')}}{1 + 10^{K(t' - t_1')}} \end{aligned} \tag{8}$$

When the two equations are thus added together, the resulting curve fits the observed data with the accuracy indicated in figures 19 and 20. The second curve reaches a lower maximum than the first and the carbon dioxide is evolved at a slower rate.

This compound curve occurs only in the nine-day data with a heavy initial inoculation of a mixed culture. Would such a second cycle curve follow all of the first cycles if incubated long enough? Would the second cycle be followed by still a third and a fourth as time progresses? Would a pure culture act similarly, or is the mixed culture responsible for the double curve? Unfortunately, our data do not answer these queries, although Figure 21 (curve IV) and figures 23 and 24 give some indication of a second cycle. But even with these the possibility remains that contamination entered into the cultures toward the close of the experiment after being opened so many times to obtain material for the cell count. Many were terminated for this reason. Furthermore, it is evident particularly in figure 23 that a higher value for *A* could be chosen which would bring the curve up

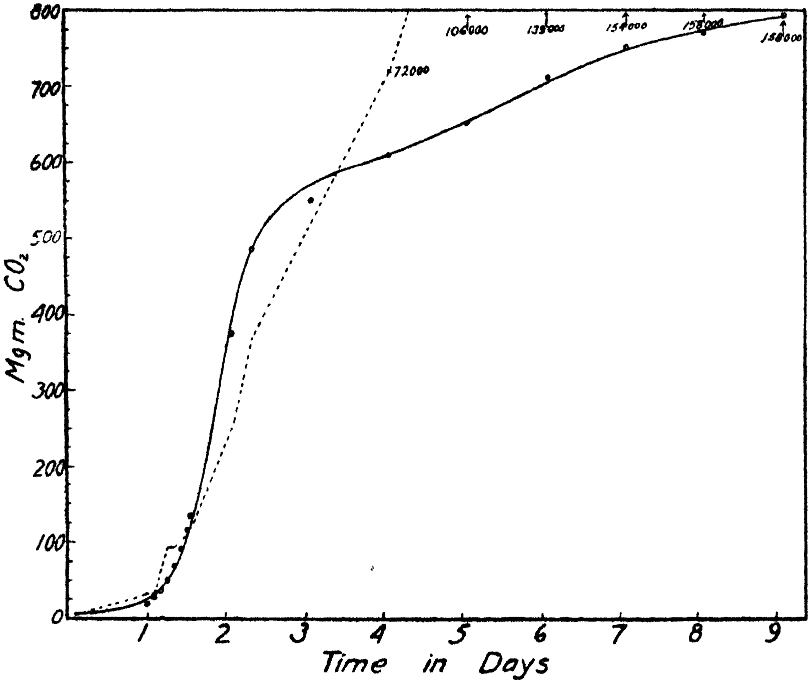

FIG. 19

through the last data points without increasing its departure from the other data points beyond experimental error, although the fit will not be as good as the one shown. Figure 21 (curve IV) is from a single flask without duplication.

The figures following figure 20 are from data using pure yeast cultures and a more limited inoculation. The incubation period

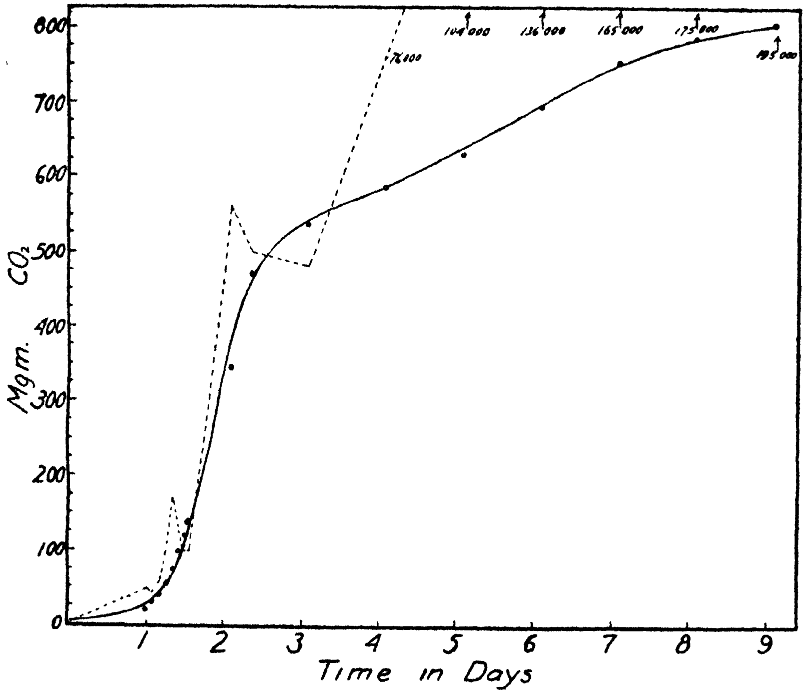

FIG. 20

was in no case shorter than seven days and in some it was thirty-one days.

The constant k decreases as the growth curve becomes longer and slower. The magnitude of A is from 250 to 900 mgm. of carbon dioxide. This varies more with the cultural media and iodine treatment than with the amount of the initial inoculation. The value of t_1 is controlled mostly by the amount of the initial inoculation, being highest where least inoculum is used.

Simultaneously with the carbon dioxide determination, a count was made of the number of cells present in the media. The broken curves on the graphic figures indicate the count obtained. It was more erratic than the carbon dioxide determination, and for this reason does not justify curve fitting. At times the count would suddenly drop, due to clumping of the cells; the numbers were also counted only above 320 thousand cells per cubic centimeter; the data record, no growth, where the numbers are fewer than this. In spite of these inaccuracies, it is evident that the multiplication of cells is most rapid during the period of most rapid carbon dioxide evolution, or immediately following it. The iodine acts as a stimulant up to a concentration of about 1000 p.p.m. and in higher concentration as a depressant. Cell multiplication is more responsive to stimulation by iodine than is carbon dioxide evolution and is also more sensitive to the toxic effects of the higher concentrations.

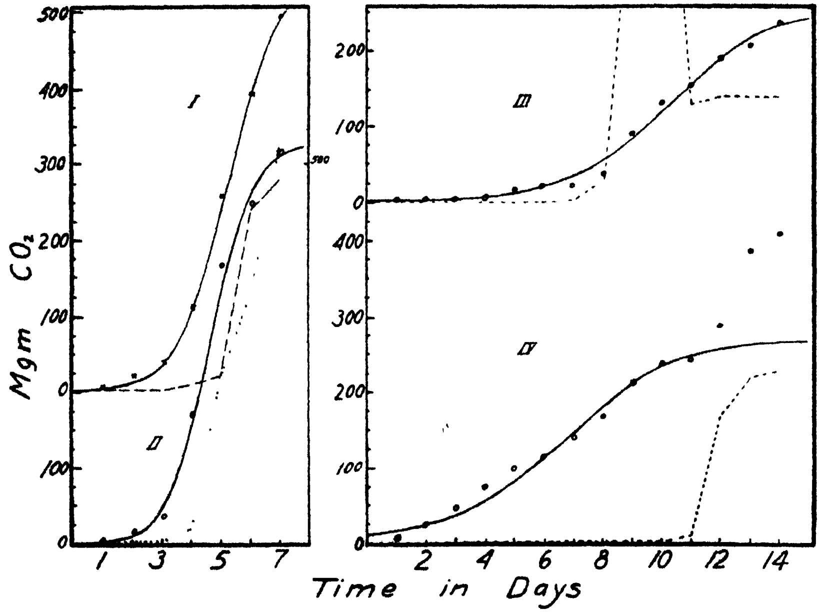

FIG. 21

We may conclude from this study that carbon dioxide is evolved according to the autocatalytic formula. Our data suggest that one curve succeeds another, the succeeding one being more prolonged and of smaller magnitude than the first. Our data on cell multiplication do not contradict other data which show that increase in numbers of cells follows the formula we have used. The increase in the number of cells is not proportional to the carbon dioxide production. The cell count will eventually reach a maximum, but the total carbon dioxide evolved will not, as long as any living cells remain. This consideration supports the idea that the evolution of carbon dioxide takes place in cycles, one following another and each decreasing in magnitude but more prolonged than its predecessor, rather than discontinuing at the definite maximum of a single-growth curve.

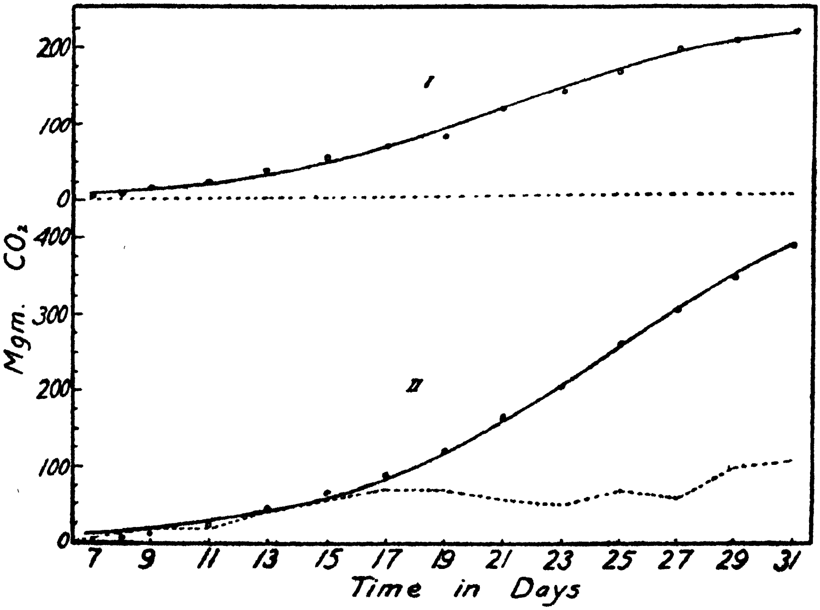

Fig. 22

Our attempt has been to show that the theory advanced by Robertson (1923) to explain the rate of growth is also applicable

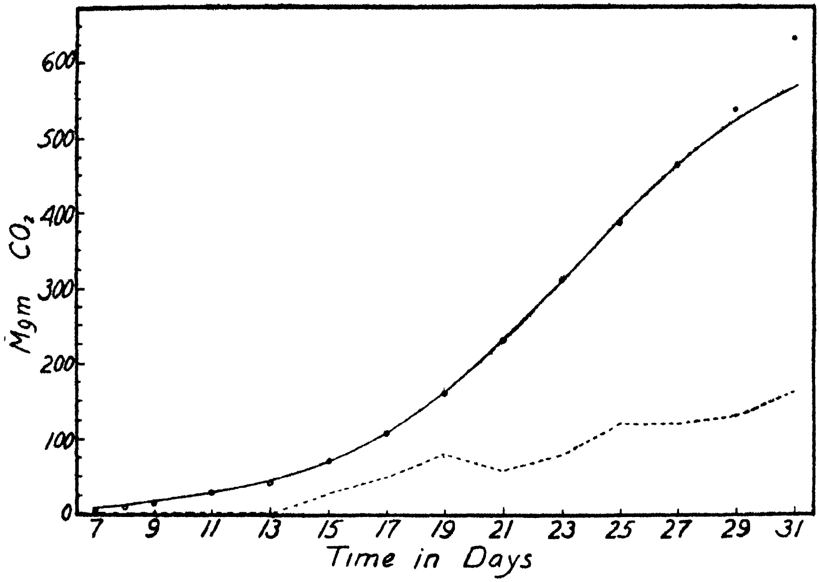
Mgm CO₂
600
500
400
300
200
100
0
7
9
11
13
15
17
19
21
23
25
27
29
31
Time in Days

FIG. 23

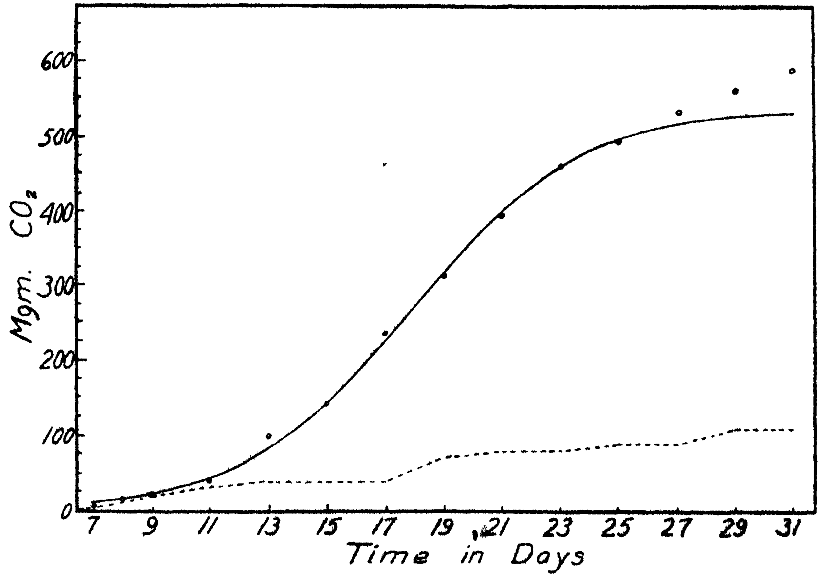
Mgm. CO₂
600
500
400
300
200
100
0
7
9
11
13
15
17
19
21
23
25
27
29
31
Time in Days

FIG. 24

to the various products of microbial metabolism. Both growth and metabolism are governed by the principle of the "master reaction." Protoplasmic synthesis is the result of a large number of interdependent chemical reactions. If any one reaction is retarded, all are retarded by a proportional amount. A measure of any one then gives a measure of the progress of all the others. It is for this reason that the laws governing the speed of a single monomolecular reaction immediately apply to the sum total of the metabolic activity. Our experimental indices of metabolism are the end products formed, e.g., total growth, nitrates, and carbon dioxide.

SUMMARY

The progress of nitrate accumulation by mixed cultures of soil bacteria and of carbon dioxide by pure cultures of *Saccharomyces cerevisiae* and by commercial yeast cultures can be defined by the equation

$$y = \frac{A \cdot 10^{K(t - t_1)}}{1 + 10^{K(t - t_1)}}$$

which is derived from the equation expressing the speed of a monomolecular autocatalyzed chemical reaction:

$$\frac{dy}{dt} = ky(a - y)$$

In the case of carbon dioxide production it is suggested from limited data that one cycle of evolution follows another, the curve being calculated by adding for the value of y the several separate values at any given time.

In the equation are two variables y, the metabolic product, and t the time, and three constants. The constant K is significant of the conditions of culture—whether favorable, stimulating or depressing. The constant t_1 is influenced most by the amount of the initial inoculation. The constant A is rather independent of environmental conditions until they become deleterious to the organisms. Then, A is notably decreased.

The carbon dioxide produced by yeasts under varying conditions is not proportional to the increase in the number of cells. Illustrative graphs are given to show the application of the above formula to experimental data.

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AN AERATION TRAIN FOR THE STUDY OF PRODUCTS OF BACTERIAL METABOLISM[1]

HAROLD H. WALKER

Department of Public Health, Yale School of Medicine

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OBJECTIVES

The chemical end-products of bacterial metabolism have been used since the early days of the science as a means of classifying bacteria; and systematic bacteriology rests in large part upon such determinations. In recent years the unique advantages offered by bacteria for the elucidation of the basic problems of cell physiology have been increasingly recognized.

The study of bacterial metabolism in the past has generally been concerned either with the products of protein metabolism (summarized by Rettger (1928)) or with the products of carbohydrate metabolism (reviewed by Kendall (1928)). In the former case, analyses of the medium give at least a fair picture of the situation. In carbohydrate metabolism, on the contrary, the acidity of the medium may, as shown by Merrill (1930) for mycobacteria, bear no relation whatever to actual decomposition of sugars. On the other hand, the study of carbon dioxide production in the Smith fermentation tube or in similar pieces of apparatus can only permit the most general conclusions. As shown by Keyes (1909) and Rogers, Clark and Davis (1914) and others, diffusion from the open surface of such a medium is so considerable and so variable a factor that the method throws little light on basic metabolic changes.

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[1] Based on a portion of a dissertation presented to the Faculty of the Graduate School of Yale University in partial fulfillment of requirements for the Degree of Doctor of Philosophy.

Presented at the Thirty-first Annual Meeting of the Society of American Bacteriologists, December 30, 1929.

Rogers, Clark and Davis (1914) themselves obtained highly significant results by collecting and analyzing all the gases produced by colon-group organisms,—results which established a clear differentiation between the *Esch. coli* and *Aero. aerogenes* types on the basis of the ratio of carbon dioxide to hydrogen in the gases which they liberate. A further advance was made by Novy, Roehm and Soule (1925) who collected the gas formed by bacteria in a compensation manometer so that the rate of gas production could be watched as well as the composition of the gas determined. Soule (1928) on the basis of this method was able to compute for several bacterial and protozoal types respiratory quotients which varied from 0.8 to 1.3.

A simple respirometer in which CO_2 produced by yeast was measured has been used by Rahn (1929, a and b) to establish time-number-product relationships, and the micro-respirometer developed by Warburg (1926) has been recently used for bacteriological investigations by Eaton (1931) and by Burk and Lineweaver (1930).

These studies have contributed very materially to our knowledge of quantitative bacterial metabolism. For the prosecution of certain investigations which are in process in the laboratories of the Department of Public Health of the Yale Medical School, we desired to attain certain ends which no previous technic made possible. Our desiderata were as follows:

a. The cultivation of bacteria in a medium which should be kept free of as large a proportion as possible of the waste products of metabolism so as to permit the study of a reasonably normal cycle. That ordinary test tube cultivation is unfavorable for optimum growth conditions has been increasingly remarked of late. Rogers and Whittier (1930) by continual ingress of fresh medium have kept *Str. lactis* and *Esch. coli* at maximum population for one month without onset of a death phase, and Magoon and Brunstetter (1930) by aerating cultures have obtained greatly increased growth.

b. The determination of the rate of bacterial growth so that metabolic activity could be correlated with definite phases of the life cycle and metabolic products correlated with the number of cells present at a given time.

c. The simultaneous determination of all the ammonia formed (as a net end-product of protein metabolism) and of all the carbon dioxide formed (as an end-product of carbohydrate metabolism),— whether retained in the medium or given off from it. It is scarcely possible to draw sound conclusions as to the so-called "respiration" of microörganisms without knowing something of ammonia production also on account of the interaction of acidic and basic substances in a complex organic medium.

The apparatus briefly presented two years ago (Walker (1930)) has proved so successful for this purpose and seems to have so many possible applications as to warrant full description. Several of its features are included in aeration methods developed at about the same time by Krishna (1928) and by Merrill (1930).

GENERAL PLAN OF APPARATUS

The general plan of the apparatus used is shown in figure 1. It involves three major sections as follows:

A culture unit in which the bacteria are grown in any appropriate medium which is constantly aerated by a current of air which has been freed from ammonia and carbon dioxide. This unit is shown at *E* in figure 1.

A purification train for removing ammonia and carbon dioxide from the incoming air. This is shown at *A–D* in figure 1.

An absorption train in which the ammonia and carbon dioxide carried off by the aerating current can be collected. This is shown at *F–H* in figure 1.

Purification train

The purification train includes four units, as follows:

Unit *A* (fig. 1). A Milligan spiral gas-washing bottle containing a strong solution[2] of KOH for the absorption of CO_2. Repeated tests showed that removal of CO_2 was consistently 100 per cent complete.

Unit *B*. A similar bottle containing a strong solution[3] of H_2SO_4 for absorption of NH_3.

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[2] Specific gravity of KOH solution approximately 1.3.

[3] Specific gravity of H_2SO_4 approximately 1.7.

Unit *C*. A bottle of distilled water (several hundred cubic centimeters) to serve as a trap preventing mechanical entrainment of acid from unit *B*.

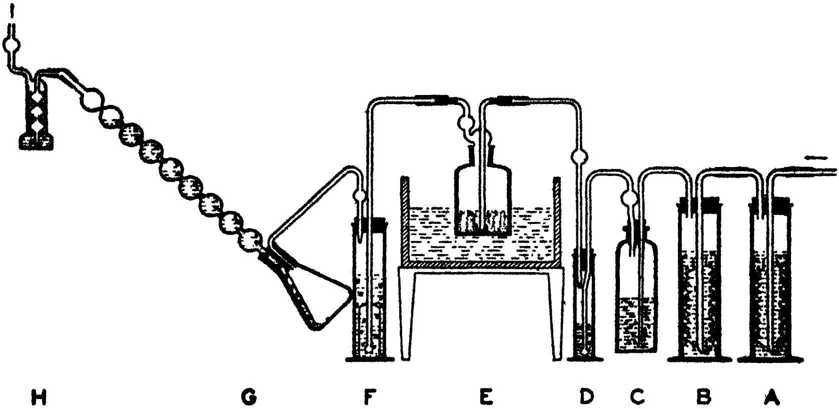


FIG. 1. TRAIN FOR REMOVAL AND DETERMINATION OF FREE CO_2 AND NH_3 FROM BACTERIAL CULTURES GROWING UNDER THE INFLUENCE OF CONTINUOUS AERATION

KEY TO FIGURE 1—AERATION TRAIN

Division I. Purification train

A. Milligan spiral gas-washing bottle.
   Contents—strong solution KOH, for removal of CO_2 from incoming air.
B. Ditto. Contents—strong solution H_2SO_4, for removal of NH_3.
C. Glass bottle.
   Contents—distilled water for trap against mechanical entrainment from *B*.
D. Small cylinder.
   Contents—distilled water and bromthymol blue or other colorimetric pH indicator, for control on atmospheric purification.

Division II. Culture unit

E. Sterile Dreschel "low form" glass-stoppered gas washing bottle, capacity 250 cc.
   Contents—100 cc. or more culture medium and organisms, for aeration during incubation in water bath.

Division III. Absorption train

F. Cylinder, 8 by 1½ inches, with Folin ammonia absorption bell.
   Contents—100 cc. 0.05 normal H_2SO_4, for absorption of NH_3 aerated from culture; results determined by neutralization and direct Nesslerization of aliquot portion.
G. Erlenmeyer flask, capacity 250 cc. and Brady-Meyer absorption tube.
   Contents—75 cc. standard solution $Ba(OH)_2$ for absorption of CO_2 from culture; results determined by titration of residual $Ba(OH)_2$, with standard HCl and thymolphthalein, CO_2 remaining precipitated as $BaCO_3$.
H. Bowen potash bulb.
   Contents—saturated $Ba(OH)_2$ for control on efficiency of absorption of Brady-Meyer tube (*G*).

Unit *D*. A test cylinder containing distilled water tinted with bromthymol blue as a check on the efficiency of units *A* and *B* in removing CO_2 and NH_3 and on the efficiency of unit *C* in trapping entrained acid. This proved to be a highly important element in the apparatus. After about a day's aeration, the principal trap bottle (unit *C*) would absorb enough acid by entrainment to begin to yield up acid to unit *D*. As soon as this occurred and the color in unit *D* began to change, the apparatus was stopped and units *C* and *D* were rinsed and replaced.

It is of interest to note that when the apparatus was working normally, the reaction in unit *D* was slightly on the alkaline side of the neutral point. This alkalinity was only maintained during the process of aeration with CO_2-free air and was presumably due to traces of alkali in the distilled water (or dissolved from the glass) made manifest by removal of CO_2,—an explanation apparently consistent with observations of Fawcett and Acree (1929).

No part of the purification train required sterilization as was shown by long-continued aeration of sterile media in control experiments. The slight tendency of bacteria to leave a liquid phase and the filtering effect of the cotton plug in the inlet of the culture unit proved amply sufficient to guard against contamination.

Culture unit

The culture unit (*E*, fig. 1) consisted of a sterile Dreschel "low form" glass-stoppered gas-washing bottle with a capacity of 250 cc. and containing a known amount (100 to 175 cc.) of inoculated culture medium. It was immersed in a water bath kept at constant temperature (37°C.) by thermo-electric regulated heat. Variations were usually within a few tenths of a degree in either direction.

Two culture units containing the same medium, each with an absorption train, were aerated in parallel from a single purification train in the water bath at the same time,—one for the culture under observation and the other for an uninoculated control. The aeration rate commonly employed in our experiments was 2.5 to 3.5 liters of purified air per hour for each *E* vessel.

The Dreschel bottles after sulphuric-dichromate cleaning, were prepared by plugging inlet and outlet arms with non-absorbent cotton and covering the arms with glass caps pushed over cotton packing. A strip of paper was inserted between the ground glass stopper and its seat and the assembly then sterilized by dry heat in excess of 160°C. for over one hour.

At the start of an experiment the sterile ingredients of the media, in solutions of suitable stock concentration, were measured aseptically according to a dilution schedule into the Dreschel bottles and control plates made from each stock solution. Two filled Dreschel bottles (one for culture and one for uninoculated control) were placed in the water bath side by side and aerated, without absorption units (*G* to *H*), for one to three hours or more, during which time they rose to the temperature of the water bath and any excess free gases were expelled.

When ready for inoculation, the Dreschel bottles were lifted from the water bath to a table and similar volumes of bacterial suspension and sterile water were added respectively to the culture and the control. After being shaken, samples for plating and for chemical medium analyses were rapidly and aseptically removed from both, the bottles returned to the water bath, aeration resumed and the absorption units connected. Similar manipulations occurred at each subsequent sampling period, except, of course, that no bacteria or water were added. Caution was exercised to keep the bottles out of the water bath for the briefest possible time.

The volume of culture in the 250 cc. Dreschel bottles was planned to approach approximately 100 cc. at the midpoint of experiments. Accordingly a starting volume of 100 to 175 cc. was employed, dependent on the amounts to be withdrawn at sampling periods. Record was kept of the number of cubic centimeters thereafter removed,—hence the volume present during any stated period was known,—and the observed gas yields for each period were multiplied by an appropriate factor to convert them to yields per 100 cc. of culture volume. The record of sample portions removed was confirmed at the close of experiments by measure of the terminal volume, which showed substantial agreement with the calculated remainder.

At the start and close of each gas collection period, the menstruum was analyzed for NH_3 and CO_2. The content of each in the control medium was then subtracted from that in the culture and the net increase in medium content for the period calculated. The methods used involved modifications of the well-known procedures of Van Slyke and Cullen (1914) and Van Slyke and Stadie (1921), respectively. The modifications were conditioned by the very minute values with which we were dealing and will be described in a subsequent communication (Walker and Winslow, 1932). To the NH_3 and CO_2 yields thus revealed by net increase in menstruum content over an observed period were added the amounts of these gases which had been continuously removed by aeration during the period, their determination being as follows.

ABSORPTION OF AMMONIA

Ammonia was absorbed in unit *F*, which was an 8 by 1½-inch cylinder equipped with a Folin ammonia absorption bell and containing 100 cc. of approximately 0.05 normal H_2SO_4. Early tests showed that anticipated yields of free NH_3 would in many instances be too small for accurate determination by titration. After neutralization, direct Nesslerization of an aliquot according the Standard Methods of Water Analysis (1925) of the American Public Health Association and American Water Works Association was therefore tried, modified by the fact that preliminary clarification was unnecessary.

The absorbent to be used was crucial since it must retain NH_3 and pass CO_2 but must not be too acid for Nesslerization. The problem was worked out with the last named criterion as a starting point. The Nessler's reagent contains a strong base necessary to proper color development. Obviously, if any acid solution were to be directly Nesslerized, arrangement must be made to leave a sufficient excess of free alkali to avoid depression of color. The critical zone for HCl or H_2SO_4 was found to lie in the vicinity of N 0.025 to 0.050. A concentration of N 0.025 could be neutralized (in relation to methyl orange) by slightly less than half the alkali contained in the amount of reagent regularly used in Nesslerization. A concentration of N 0.050 was not neutralized by one-half the reagent but was rendered alkaline on addition of the second half. Tests were then made by adding standard NH_4Cl to acids, Nesslerizing, and comparing the developed color with that in acid-free standards prepared from the same NH_4Cl solution. The results showed that N 0.050 HCl partially but definitely suppressed color development, while N 0.005 had no deleterious effect. However, it was further learned that by neutralizing N 0.050 acid with NaOH prior to adding the Nessler's reagent, inhibition of color could be avoided.

It was deemed inadvisable to attempt using stronger acids, for alone they would seriously exceed the tolerance of the Nessler's reagent and, if neutralized, they might introduce additional error. Therefore it was tentatively resolved to absorb the aerated ammonia in N 0.05 HCl or H_2SO_4 and to determine the amount by direct Nesslerization, the tubes during dilution, and before addition of the reagent, to receive an amount of 5 N NaOH equal to one-hundredth the volume of the acid aliquot sampled.

TABLE 1

Series-absorption of aerated NH_3 from peptone cultures of Esch. coli

ABSORBENTS, 2 IN SERIES	LENGTH OF AERATION	NH_3-N FOUND	
		First absorbent	Second absorbent
	hours	*mgm.*	*mgm*
100 cc. N 0.05 H_2SO_4	16	0.696	<0.003
100 cc. N 0.05 HCl	23	0.560	<0.010
100 cc. N 0.50 HCl	72	11.000	<0.004

Obviously, the next procedure was to prove that N 0.05 acid would absorb NH_3 completely, would not appreciably retain CO_2 and would not exhibit volatility or mechanical entrainment deleterious to the $Ba(OH)_2$ in the succeeding unit (*G*). To test these points a series of experiments were performed.

Adequacy for NH_3 absorption was proven by continuously aerating the gases from an incubating peptone culture of organisms (*Esch. coli*) into two (*F*) units in series, each containing 100 cc. of N 0.05 acid. The results of two such tests and of one with stronger acid are presented in table 1.

It is evident that a high degree of absorptive efficiency was obtained. In the light of a large body of later experience it seems probable that the amounts of ammonia found in the second serial absorbents as shown above do not represent a carry-over from their predecessors, since they are of a magnitude appearing in almost any use made of this method and may be related to absorption from rubber stoppers or connecting tubes.

Non-retention of CO_2 by the acids was established in two ways. First, gases produced by a culture of organisms in standard lactose broth were passed for nineteen hours through an (*F*) unit containing 100 cc. N 0.05 HCl. It is probable that in this time about 100 mgm. of CO_2 were blown through the cylinder. The culture was then cut out of the line and air from the purifying train passed at a very brisk rate directly into the cylinder, which was now followed by an absorbent vessel containing saturated $Ba(OH)_2$. In one-half hour no turbidity appeared. After removing half the contents of the cylinder for Nesslerization, the remaining 50 cc. were briefly aerated to remove any room atmosphere absorbed during withdrawal of the first half. The saturated $Ba(OH)_2$ was again connected, sufficient strong H_2SO_4 added to the N 0.05 HCl to make a concentration of nearly 20 per cent acid and air was then blown rapidly through the entire assembly for eight hours. The barium hydroxide remained free from turbidity.

The second method of testing non-retention of CO_2 was as follows: The gases from an actively growing culture of *Esch. coli* in 1 per cent peptone + 0.1 M NaCl were aerated for two hours through an (*F*) unit containing 100 cc. N 0.05 H_2SO_4. Analysis of the succeeding unit (*G*) showed that 16.4 mgm. CO_2 had passed through *F* in this time. Simultaneously, air from the same purifying train had been passed into a parallel (*F*) unit through an equal volume of peptone medium containing no organisms. Aliquots from the two (*F*) units, which had therefore respectively experienced aeration with CO_2-containing and CO_2-free air, gave identical readings for dissolved gases when analyzed for CO_2 in a Van Slyke blood gas apparatus.

In addition to non-interference with Nesslerization, efficiency for NH_3-retention, and non-retention of CO_2, three other properties of unit (*F*) were controlled. Possibility of interference with the following alkaline unit (*G*) was first tested. To this end, purified air was passed successively through a culture bottle containing sterile distilled water, then through an (*F*) unit containing N 0.05 acid, then through a tube of distilled water and in some cases an (*H*) unit with saturated $Ba(OH)_2$. At the end of the aeration period, brom thymol blue was added to the distilled water which followed the (*F*) unit. In some experiments, the aeration was maintained for twenty hours and in others for ninety hours, but no blown-over acidity was detected by the brom thymol blue from either N 0.05 H_2SO_4 or N 0.05 HCl. As points of additional interest, the (*F*) units and the sterile distilled water flasks were Nesslerized and the saturated $Ba(OH)_2$ terminal units watched for turbidity due to $BaCO_3$ or $BaSO_4$. The negative results of all these blank runs proved the efficiency of the purifying train while confirming the non-volatility or non-entrainment of the NH_3 absorbents at the aeration rate employed.

The possibility of false positive NH_3 results due to entrainment of culture medium into unit (*F*) was next studied by aeration of sterile media. Results showed that only very small amounts of ammonia could be traced to this source from peptone and lactose-peptone media in a much longer period of aeration than was actually used in our metabolism studies.[4] From Dolloff's medium, which contained 76.5 mgm. of NH_3-N per 100 cc., more definite amounts were aeratable.[5] Since no method to prevent this could be discovered and since all yields from metabolism studies were based on subtraction from observed culture products of amounts obtained from parallel and simultaneous aeration of sterile control media, this matter had to be allowed to rest with the latter type of control.

The final factor investigated was the NH_3 content of the sul-

[4] In five tests aeration of sterile 1 per cent peptone followed by dilute acid absorbents revealed that in time periods as long as twelve to twenty hours the average amount of NH_3 carried over from the medium would not be expected to exceed 0.001 to 0.007 mgm.

[5] In 4 experiments aeration of sterile Dolloff's medium for twenty-four hours into N 0.05 H_2SO_4 showed an average of 0.129 mgm. of NH_3-N carried over.

phuric acid itself. The amount of NH_3–N in a routine determination attributable to this source was found to be between 0.002 and 0.004 mgm. per 100 cc. of N 0.05 H_2SO_4 absorbent. Thereafter the amount equivalent to this content, in cubic centimeters of standard NH_4Cl, and proportionate to the aliquot acid Nesslerized, was deducted from all readings as observed against the color standards.

In Nesslerization, fresh color standards were prepared when used in preference to keeping permanent standards. The distilled water was collected from a Barnstead still and stored in 6-liter Pyrex flasks equipped for siphon delivery, the incoming air being drawn through a tube of sulphuric acid to exclude atmospheric NH_3. This water gave a very faint color reaction with Nessler's solution which may have been largely dilution of the color of the reagent, since an addition of standard solution to the extent of only 0.001 mgm. of NH_3–N produced a visible color response. The 50 ml. color comparison tubes conformed to the American Public Health Association Committee's specifications.

ABSORPTION OF CARBON DIOXIDE

Carbon dioxide absorption was accomplished in units *G* and *H*. Unit *G* was a 250 cc. Erlenmeyer flask equipped with a Brady-Meyer absorption tube. This device is used in the steel industry for the determination of carbon (Brady, 1914). The CO_2 absorbent was standardized $Ba(OH)_2$ of about N/10 concentration. Twenty-five or 50 cc. were measured by a burette into the Erlenmeyer flasks and CO_2-free distilled water added to make the final volume 75 cc., the amount needed for proper occupancy of the Brady-Meyer bulb tube. The culture and control flasks were kept tightly stoppered between filling and use, at which latter time the bulb-tubes and connecting tubes, assembled in their 2-hole stoppers, were introduced. When aeration was completed, the fluid was allowed to return to the flasks from the bulb tubes, the latter being then carefully rinsed down with 40 cc. CO_2-free water from a protected siphoning reservoir. The flasks were tightly stoppered and set aside for subsequent titration. Traces of atmospheric CO_2 probably gained entrance in the filling or rinsing down processes or by stopper perfusion but since results were reported as differences between cultures and controls and the parallel flasks were in all manipulations given the same handling, it is unlikely that serious error occurred from such sources.

The amount of CO_2 absorbed was determined by back titration of the free $Ba(OH)_2$ remaining unprecipitated, the volume of which was deducted from the original $Ba(OH)_2$. The advantages of thymolphthalein as an indicator for this type of titration were pointed out by Schollenberger (1928). Its use has been found very satisfactory.

The permanent standard for this part of the work was N/10 HCl, prepared by volumetric dilution from a purified stock solution, the strength of which had been fixed by distillation at constant boiling point. The final figure for its value was based on an ultimate standard of carefully weighed portions of dried potassium acid phthalate titrated through an intermediary of CO_2-free NaOH. The permanent N/10 HCl gave in terms of the phthalate, on two standardizations, made a year apart by different laboratory workers, values of N 0.1029 and N 0.1028.

The $Ba(OH)_2$ solution was prepared by dissolving weighed crystals in boiling CO_2-free distilled water and was clarified by filtration into a volumetric flask freed of atmospheric CO_2 by aeration. When cooled and diluted with CO_2-free water the solution was stored in a large reservoir equipped with a Squibb's automatic-O type burette. Soda-lime tubes were placed on the air vent arm of the burette and between the reservoir and the rubber bulb supplying air pressure. The solution was then standardized in terms of its volumetric ratio to a known working solution of N/10 HCl, using the mean of values obtained in titrating the acid with the alkali in the presence of two indicators (alizarine sodium monosulfonate and phenolphthalein) lying close on either side of true neutrality. A minor error which would have been incurred in making the subsequent experimental titrations with thymolphthalein, the end point of which is near pH 10, was avoided by the subtraction of the blank from the sample. The $Ba(OH)_2$ solution as stored in the protected

Squibb's burette was found to retain its strength very satisfactorily.

The distilled water used in all CO_2 work was prepared as follows: Atmospheric CO_2 was expelled from a clean 6-liter Pyrex flask by aeration with CO_2-free air. Meanwhile, the Barnstead still with dissolved gases escaping in the steam waste exhaust was set in operation and the early yield discarded. A 2-hole stopper with short straight inlet tube and a capillary outlet tube was connected directly to the discharge from the still. The condensed water was thus collected with avoidance of serious exposure to room atmosphere, since it was presumed that little back diffusion would occur through the capillary outlet from which air and hot water vapor were continually displaced by the collecting distillate. After being cooled the flask was stored for siphon delivery by a soda-lime protected burette. The air intake for the flask was protected by a tube of strong NaOH solution. Water from this reservoir would for many days exhibit a pH of 6.9 to 7.1 with brom thymol blue if tested as soon as drawn and without shaking. In the light of the recent work of Fawcett and Acree (1929) it seems possible that the above procedure may not have yielded a strictly CO_2-free water, but it always appeared satisfactory for our purposes.

One other feature was given attention. This was the question of whether the Erlenmeyer flasks (unit *G*) containing the experimental yields could be safely titrated in the open or would require passage of a stream of CO_2-free air while being held under the burette. The possibilities were that the unprotected $Ba(OH)_2$ might absorb atmospheric CO_2, or that the aerated $Ba(OH)_2 + BaCO_3$ might lose CO_2 during titration. The results of trial titrations under several conditions indicated that flasks of $Ba(OH)_2$ diluted proportionately to those employed in the metabolism studies, whether containing $Ba(OH)_2$ or $Ba(OH)_2 + BaCO_3$, could be titrated unstoppered, with gentle shaking, without recourse to the aeration technic. This more common procedure was thereafter followed.

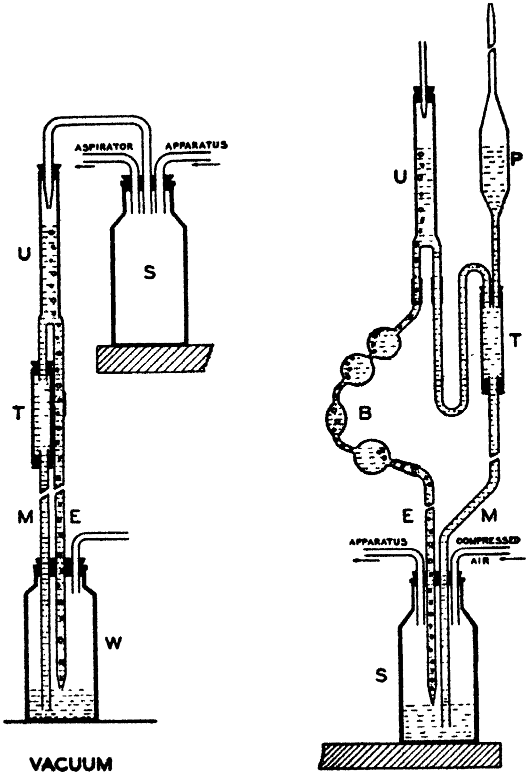
The final aeration unit (*H*), a Bowen potash bulb containing saturated $Ba(OH)_2$, served to control completeness of absorption in the preceding unit (*G*). So long as the standard solution in (*G*) was not overloaded by CO_2 its absorption performance was shown by this control to be 100 per cent complete. In metabolism experiments in which over 100 mgm. of CO_2 were harvested in unit *G* no evidence appeared of any carry-over to unit *H*. The similar bulb at the end of the aeration train containing the sterile control medium served incidentally as a constant control on the freedom from CO_2 of the air which was being fed by a *Y* junction from the one purification train (*A–D*) into the parallel culture and control trains (*E–H*).

SAFETY VALVES

For use with our aeration, which was supplied by compression from a laboratory tap, considerable preliminary time was spent in perfecting a safety valve which would satisfactorily permit escape of excess pressure without permitting kick-back of fluids in the long train. The valves finally developed are shown in figure 2. In all our later work only the pressure valve (right) was employed. The principle underlying the valves is evident from the figure and its key. This part of the apparatus has been highly satisfactory in that it not only prevented kick-back but has even permitted aeration through the train to continue while excess pressure was escaping. Many an experiment was thereby saved from accidental ruin.

The complete aeration train was operable under a head of about 3 feet of water (equal to 3 inches of mercury) in the safety valve. The usual rate of aeration was 2.5 to 3.5 liters of air per hour per culture flask.

The rubber stoppers and tube connections employed in our apparatus were cleaned after the method recommended by Novy, Roehm and Soule (1925), which involved boiling in dilute alkali and in acid, thorough rinsing, and autoclaving in glycerol, after which they were preserved in glycerol to insure a moist, tight seal. Whenever possible, glass junctions were kept end against end in order to minimize rubber surface exposure.



VACUUM

PRESSURE

FIG. 2. SAFETY VALVES DESIGNED FOR USE WITH AERATION TRAIN

KEY TO FIGURE 2—SAFETY VALVES FOR AERATION TRAIN

	Function	*Composition*
S	Safety bottle and pressure seal	Large-mouth, heavy glass bottle
S	Vacuum safety bottle	Large-mouth, heavy glass bottle
W	Vacuum water seal	Large-mouth, heavy glass bottle
E	Pressure escape column	Glass tubing, constricted tip
M	Pressure maintenance column	Glass tubing
U	Common reservoir uniting (*E*) and (*M*)	Test tube 8 inches by 1 inch with *U*-base inlet tubes
T	Trap against air rebound	Glass tube, 5 inches by 1 inch
P	Pressure maintenance reservoir	100 cc. pipette, inverted
B	Escape air-arresting bulbs	Potash bulb

S may rest on table with apparatus.
W may rest on floor.
Supporting stands and clamps not shown.

SUMMARY

A method has been described which makes it possible to cultivate bacteria in a medium continuously aerated by a stream of air which has been freed from ammonia and carbon dioxide; and to determine with accuracy the yielded amount of both ammonia and carbon dioxide in the air which has passed through the culture. The pitfalls in such a procedure are so many that it has seemed justifiable to describe in considerable detail the methods as finally worked out. The determination of the ammonia and carbon dioxide remaining in the culture medium can be made by slight modifications of standard technics used in the physiological laboratory which will be described in a subsequent communication (Walker and Winslow (1932)).

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THE INFLUENCE OF AERATION AND OF SODIUM CHLORIDE UPON THE GROWTH CURVE OF BACTERIA IN VARIOUS MEDIA[1, 2]

C.-E. A. WINSLOW, H. H. WALKER AND MARGARET SUTERMEISTER

Department of Public Health, Yale School of Medicine

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SCOPE OF STUDY

In the course of studies on bacterial metabolism conducted in the Department of Public Health of Yale University, it became necessary to establish certain standard bacterial growth curves, so that periods could be selected which would represent with accuracy the lag phase, the phase of logarithmic increase and the maximum stationary phase (Buchanan (1918); Buchanan and Fulmer (1928)) or phase of peak stability (Winslow (1928)).

Four different media were to be employed in the metabolism studies and the influence of these four media constituted the first phase of the research. Secondly, the metabolism observations were to be made in a medium constantly aerated with air freed from ammonia and carbon dioxide; the influence of this aeration must therefore be determined. Finally, the effect of stimulating and toxic salt concentrations upon metabolic products was an essential part of the problem; and the influence of these salt concentrations upon the growth curve must be accurately known.

The determination of growth curves for these various conditions threw such light on certain phenomena related to the life cycle of bacterial populations and the effect upon this cycle of a

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[2] Based on an essay submitted by one of the authors (M. S.) to the Faculty of the Graduate School of Yale University in partial fulfillment of requirements for the Certificate in Public Health.

mineral salt that it has seemed worth while to present the results in the present paper.

TECHNIC

All the work here reported was carried out with the same strain of *Esch. coli* (*communis*) employed in previous studies from this laboratory (Cohen (1922); Fabian and Winslow (1929); Falk and Winslow (1926); Hotchkiss (1923); Winslow and Haywood (1931); Winslow and Dolloff (1928); Winslow and Falk (1923a and 1923b)). It was maintained on nutrient agar slants at 32° to 37°C., transferred every twenty-four hours and rejuvenated once a week by passage through meat infusion agar. When a very heavy inoculum was needed (with toxic salts) the organism was grown on agar in a flat bottle, similar to a Kolle flask.

The strain showed, throughout, both smooth and rough colonies. When the experimental media were first inoculated, the ratio of *S* to *R* types was generally between 0.4 and 1.1. At the end of twenty-four hours' cultivation in the experimental liquid medium the ratio of *S* to *R* colonies had generally risen to 0.5 to 2.0.

Four basic media were used, as follows: standard lactose peptone broth (0.5 per cent Difco bacto-peptone, 0.5 per cent Pfanstiehl c.p. lactose hydrate and 0.3 per cent bacto-beef extract); lactose-peptone water (the same as the standard lactose broth except for the omission of meat extract); peptone water (1 per cent Difco bacto-peptone); and the Dolloff synthetic medium (5 grams ammonium tartrate, 5 grams Pfanstiehl c.p. lactose, 0.02 gram dibasic ammonium phosphate per liter). A solution of each ingredient was prepared in higher concentration, and separately sterilized.[3] These separate solutions were then aseptically mixed to produce the media ultimately desired. In the salt experiments Baker's analyzed c.p. NaCl was added in appropriate amounts from 2.5 to 5.0 molar stock solutions. Lactose solutions were sterilized at 10 pounds for ten minutes, all others at 15 pounds for twenty minutes.

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[3] In the case of Dolloff's medium, the ammonium salts were prepared in one solution and the lactose in another.

The reactions of the standard lactose peptone broth, the lactose-peptone water and the peptone water were consistently close to pH 6.8. Dolloff's medium gave readings near pH 6.1, slightly higher than those reported by Fabian and Winslow (1929) and much higher than those reported by Winslow and Dolloff (1928). This result may be due to the fact that we sterilized the ammonium salt and the lactose portions separately and sterilized the lactose portion at lower pressure and for a shorter period.

Growth curves were determined in 30 cc. quantities of medium in 200 x 25 mm. cotton-plugged test-tubes in the case of unaerated cultures, and in 100 cc. quantities in Dreschel bottles in the case of aerated cultures. The aerated and unaerated cultures were run at the same time, both receptables being immersed in a water bath at 37°. The aerated cultures were fed by a stream of air which had been freed from NH_3 and CO_2 by the use of the aeration train described by Walker (1932) in a previous contribution. The temperature and aeration conditions were stabilized by at least half an hour's exposure of the medium to the desired conditions before inoculation.

In conducting an experiment, a twelve-hour nutrient agar slant was washed off with 10 cc. sterile distilled water and the resulting suspension filtered through sterile filter paper and diluted with a fixed amount of sterile distilled water selected so as to give the desired concentration. This concentration was such that when 3 cc. of the water suspension were added to 27 cc. of medium (unaerated) or 10 cc. were added to 90 cc. of medium (aerated), the initial count would be about 10 to 15 million per cubic centimeter. It was found by plating that with agar slants of standard size this concentration was so generally constant that comparison with turbidity standards was unnecessary. With toxic salt concentrations, the suspension was washed off from a large agar surface in a flat bottle and the dilution adjusted to give a much higher initial concentration of bacteria, often amounting to several hundred millions per cubic centimeter. Where these very heavy inoculations were used, the cells were washed by centrifuging two or three times for twenty to thirty minutes.

Portions of the cultures were withdrawn at once after inoculation and at subsequent intervals, for bacteriological counts and determinations of hydrogen ion concentration.

Plates were made in triplicate on standard nutrient agar, incubated for forty-eight hours at 32° to 37° and counted in a Buck colony counting box. Purity of cultures was checked at frequent intervals by making Endo plates or streaks.

The hydrogen ion concentration was determined by the electrometric method using the quinhydrone electrode. A Leeds and Northrop simplified type potentiometer, graduated in units of 0.0005 volt was used. The galvanometer was a Leeds and Northrup enclosed lamp and scale type. The readings were based on an Eppley standard cell. The reference solution, 1/20 M potassium acid phthalate, was freshly made every two weeks, by diluting a 1/5 M stock solution. This stock solution was made by accurately weighing the phthalate, and dissolving it in distilled water in a Pyrex bottle. The stock solution was checked by titration. In some instances where the alkalinity of the specimen exceeded the limits of the electrometric method used, the pH was determined by LaMotte color standards, using a color comparator block to allow for color and turbidity.

Altogether, 116 different experiments were made, from 1 to 10 growth curves being run for each medium and salt concentration, with or without aeration. All in all, this involved the pouring of some 5000 plates. The results presented in this paper are averages of the results obtained for each set of conditions at each period.

For one condition there was but a single experiment; for 6 conditions, 2 experiments; for 3 conditions, 3 experiments; for 5 conditions, 4 experiments; for 2 conditions, 5 experiments; for 1 condition, 6 experiments; for 3 conditions, 7 experiments; while 3 conditions were represented by 8, 9 and 10 experiments, respectively.

Individual growth curves under the same conditions checked each other very closely as indicated by table 1 which is taken at random from our data. Only in the case of the Dolloff medium with aeration were the results more variable. All subsequent tables give average results of the experiments made under a given condition.

TABLE 1

Growth of Esch. coli in 1 per cent peptone at 37°C. unaerated

Millions per cubic centimeter

AGE	INDIVIDUAL EXPERIMENTS					AVERAGE
	(1)	(2)	(3)	(4)	(5)	
hours						
0	12	12	8	17	13	13
1	11	10	19	19	15	15
2	20	20	33	51	37	32
3	41	43	98	102	74	72
4	105	80	157	140	126	122
5	108	132	139		159	135
6	133	142	168		221	166
8	154	172	223	215	145	182
10	148	163			202	171
15				227	215	
24	126	144	257	269		199
45				134		

TABLE 2

Average bacterial count in various media, aerated and unaerated

Millions per cubic centimeter

HOURS	STANDARD LACTOSE PEPTONE BROTH		LACTOSE-PEPTONE WATER		PEPTONE WATER		DOLLOFF MEDIUM	
	Aerated	Unaerated	Aerated	Unaerated	Aerated	Unaerated	Aerated	Unaerated
0	13	13	11	9	13	13	9	8
1	12	15	14	13	13	15		
1 3				15				
1 7				20				
2	60	48	22	27	34	32	10	12
3	226	127	98		153	72		
3 3				73				
4	555	180	294		430	122		12
4 7				98				
5	861	201	613		836	135	10	
6	776	251		122	1,150	166		
7			1,050			182		
8	922	183			1,750		8	16
8.5				134				
10						171		48
15	880	193			2,170		8	132
24				123	2,070	199	7	155
48								165
72								169
96								175

GROWTH CURVES IN VARIOUS BASIC MEDIA

We may first discuss the characteristics of the growth curves in the four basic media without aeration. The average results obtained, with and without aeration, are presented in table 2 and the curves in the four media without aeration are graphically presented in figure 1.

In this and subsequent graphs the abscissae represent linear time in hours and the left ordinates represent logarithms of millions of bacteria per cubic centimeter. This is the method of plotting recommended by Buchanan (1928) as bringing out the relations of growth phases most clearly.

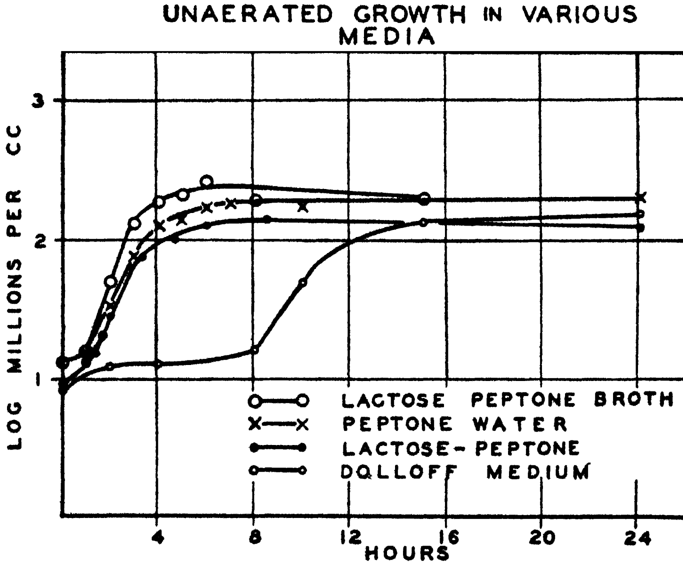

FIG. 1. CURVES OF AVERAGE GROWTH WITHOUT AERATION IN FOUR BASIC MEDIA

It will be noted from figure 1 that the curves for the media containing peptone with or without lactose are nearly identical,—although the curve for the lactose-peptone water falls slightly below the other two. All show a lag period of about one hour, a rapid and approximately logarithmic increase from the first to the third or fourth hour, and a level of approximate stability soon after the sixth hour. Our experiments were not sufficiently prolonged to cover the succeeding phases of decrease in numbers which would, no doubt, have ultimately followed.

The Dolloff medium, on the other hand, shows a very different picture. Here, the lag period continues for eight hours and the period of logarithmic increase falls somewhere between the eighth and the fifteenth hour. The longer lag period here is no doubt due to the low nutrient value of the medium, a point which will be further elucidated in connection with discussion of the effects of aeration.

The generation time formula, whose basis is attributed to Buchner, Longard and Riedlin (1887), was used to compute the minutes required during the phase of logarithmic increase for a single cell to mature, divide and produce two new individuals. This formula as expressed by Buchanan and Fulmer (1928) is as follows:

$$g = \frac{t \log 2}{\log b - \log B}$$

where g = length of one generation period in minutes,
t = duration of period of observation in minutes,
B = number of bacteria at beginning of period t,
b = number of bacteria at close of period t.

The generation times thus computed for the unaerated media (using a two-hour period in the logarithmic phase) were as follows:

	minutes
Standard lactose peptone broth	39
Lactose-peptone water	53
Peptone water	53
Dolloff	75

The richer the medium, the more rapid is the rate of logarithmic increase. It will be recalled that the standard lactose peptone broth contains lactose, peptone and meat extract. Its generation time is thirty-nine minutes. The omission of the meat extract increases the time to fifty-three minutes. In the peptone medium with no sugar, it is the same, fifty-three minutes; in the simple synthetic medium, with only lactose and ammonium salt nitrogen available, it rises to seventy-five minutes.

The length of the logarithmic period observed by us is less than that reported by Sherman, Holm and Albus (1922) and Salter (1919),—no doubt because we used a larger initial inoculum.

The reasons why the logarithmic growth phase of a bacterial cycle ends and passes into the phase of peak stability are still somewhat obscure, (Butterfield (1929); Rogers and Whittier (1930); Barnes (1931)). Either exhaustion of nutrient materials, accumulation of waste products or some other unknown factors may play a part. This problem, too, will be considered in connection with our aeration studies.

INFLUENCE OF AERATION UPON THE POPULATION CYCLE

We may pass next to the comparison of growth in aerated and unaerated media. The basic population data are included in table 2 and are presented, with parallel pH values in figures 2 to 5.

The influence of aeration may conveniently be considered with respect to the three phases of the population cycle which are represented.

The lag period is slightly but distinctly prolonged in all of the three complex media. In the Dolloff medium (fig. 5) it is prolonged for the entire period of comparative observation, no substantial change taking place in the numbers of bacteria present for twenty-four hours. Of the other three media, lactose-peptone water showed the most pronounced increase of the lag period.

It is of particular interest to note the results obtained in 1 experiment with the aerated Dolloff medium in which the aeration train got out of order. Up to the eighth hour the culture proceeded normally, with a constant population ranging between 4 and 5 million cells per cubic centimeter. During the night, between the eighth and fifteenth hour, aeration was stopped for an unknown period. At the fifteenth hour the count was still low (3 million) and aeration was resumed. The period of accumulation of products during the night had done its work, however. In spite of subsequent continuous aerating the counts went up to 9 million at the twenty-fourth hour and 215 million at the thirty-

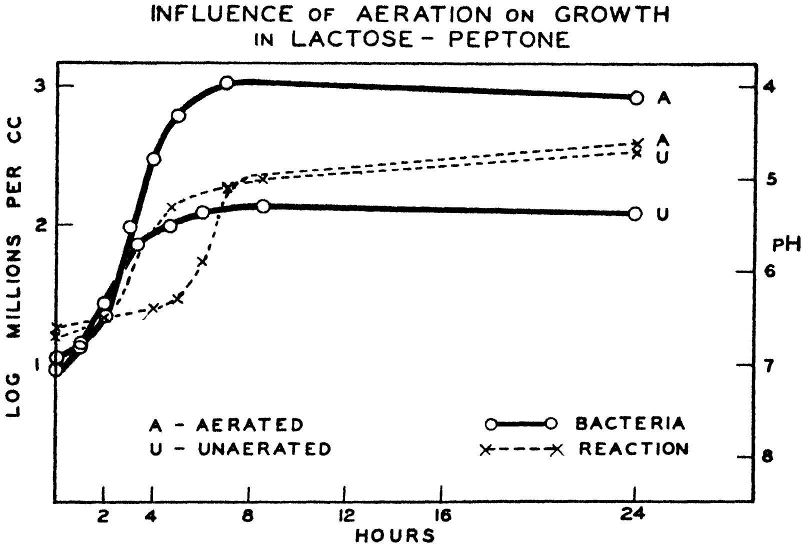

Fig. 2 Curves of Average Growth and Reaction in Aerated and Unaerated Lactose-peptone Water

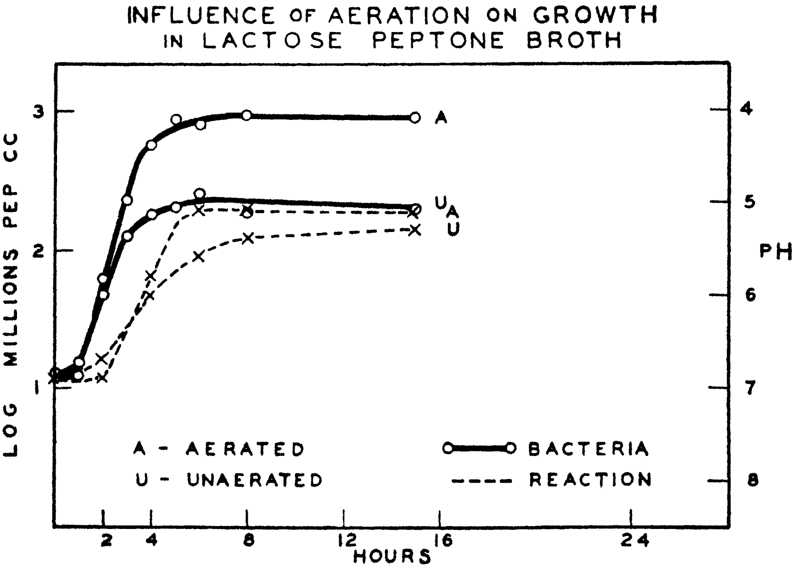

Fig. 3. Curves of Average Growth and Reaction in Aerated and Unaerated Lactose Peptone Broth

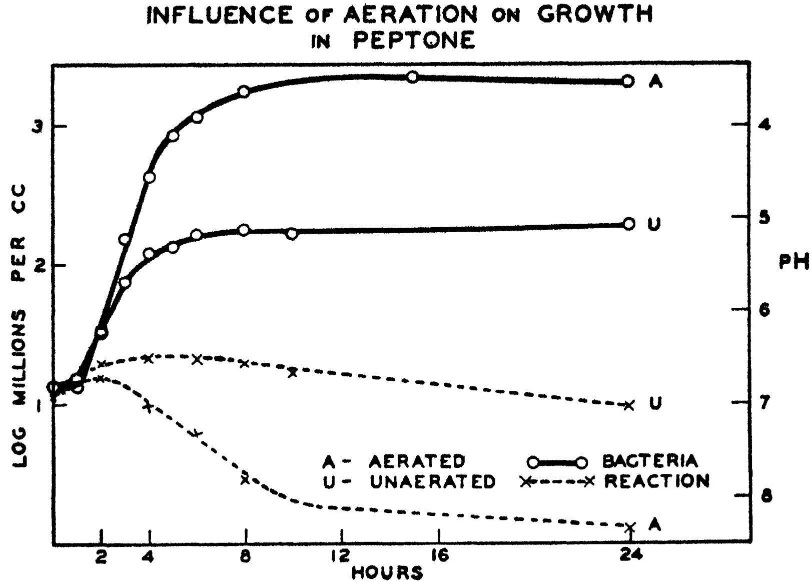

FIG. 4. CURVES OF AVERAGE GROWTH AND REACTION IN AERATED AND UNAERATED PEPTONE WATER

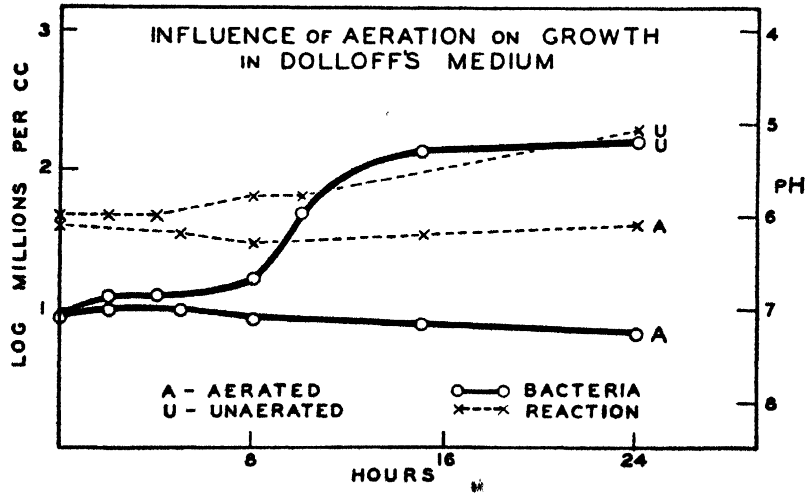

FIG. 5. CURVES OF AVERAGE GROWTH AND REACTION IN AERATED AND UNAERATED DOLLOFF MEDIUM

second hour. Two other cultures incubated with continuous aeration showed no increase for one and one-half to two days but revealed substantial growth peaks on the third day. For the sake of maintaining the Dolloff graph on a scale comparable to the others, only the twenty-four hour period of no increase has been plotted.

The lag period of the bacterial population cycle (Buchanan and Fulmer (1928); Winslow (1928)) appears whenever bacteria are transferred from one medium to another,—except when cells from a culture in the logarithmic phase are transferred to a medium of essentially the same composition. This exception to the general rule would seem to furnish the key to the whole problem. It suggests that cells in the phase of logarithmic increase are in a special biological state of adaptation to a particular medium (Barber (1908); Chesney (1916); Penfold (1914)). That this "adaptation" involves the presence of intermediate bodies which play a part in the synthesis of protoplasm is the suggestion of Penfold (1914); and the observations of Chesney (1916), Penfold (1914), Rahn (1906) and Rettger (1918) make it probable that these intermediate bodies are present in the menstruum surrounding the cells.

The effect of aeration in increasing the lag phenomena may be reasonably attributed to the removal of such "intermediate bodies." It may, however, be equally well explained by increased oxygen supply or by the removal of CO_2, in view of the demonstration by Valley and Rettger (1926; 1927) that CO_2 is a basic essential for bacterial growth. Apparently either "intermediate bodies" or CO_2 or both must be produced by slowly growing cells in the menstruum before rapid increase is possible. Aeration with CO_2-free air delays this accumulation,—in the case of the Dolloff medium for a long period. Soule (1928) has reported failure to inhibit bacterial growth by surface aeration with CO_2-free air but our experiments show that with a medium of minimal nutrient value like the Dolloff medium this result can be realized by aeration of the medium itself.

We may next pass to the effect of aeration upon the logarithmic phase. In Dolloff medium, there was of course no such phase in the aerated culture during the period studied. In the richer media, the effect of aeration, once the lag period was passed, was to increase the rate of logarithmic increase and to prolong the period of such increase. The net results are indicated in table 3.

In each instance the duration of the period of logarithmic increase is prolonged from two hours to at least three hours. The generation time is reduced by 32 per cent in peptone, by 28 per cent in lactose-peptone water and by 15 per cent in standard lactose peptone broth. As a result, the count at six to seven hours is 7 to 9 times as great in the aerated as in the unaerated culture with lactose-peptone and peptone media and three times as great with the standard lactose broth medium.

TABLE 3

Effect of aeration upon growth in various media

	STANDARD LACTOSE PEPTONE BROTH		LACTOSE-PEPTONE WATER		PEPTONE WATER	
	Unaerated	Aerated	Unaerated	Aerated	Unaerated	Aerated
Length of logarithmic phase, hours	2	3+	2	3	2	3+
Generation time, minutes*	39	33	53	38	53	36
Count, after 6 to 7 hours, millions per cubic centimeter	251	776	122	1,050	174	1,150

* The generation time values have been calculated over that span of three hours on each aerated curve (two hours on each unaerated curve) which appeared to be the most nearly a logarithmic straight line.

These results clearly indicate the value to an actively growing culture of a process of aeration which presumably operates by the removal of inhibiting waste products of growth and at the same time offers increased oxygen supply. This is in line with the work of Magoon and Brunstetter (1930) on aeration and with that of Rogers and Whittier (1930) on cultivation in continuously-flowing broth. It readily reveals the abnormal nature of the conditions under which bacteria are ordinarily cultivated in test tube studies.

It will be noted that in figures 2 to 5 we have plotted not only the population curves but also the corresponding pH values. As in the case of the counts, these pH values represent averages of the several experiments run under a given condition. While it is theoretically unsound to average figures which (like pH values) represent logarithms, the differences between the results of parallel experiments were so slight that the procedure can here involve no significant errors.

In both the lactose-peptone water and the standard lactose peptone broth (figs. 2 and 3) we note the pH curve in the aerated medium rises more slowly than in the unaerated medium, but later crosses over and reaches a slightly higher level. In each case the logarithmic phase ceases soon after the reaction reaches a pH of about 6. The better development in the aerated medium seems, therefore, to be correlated with the early removal of acidic products, primarily, in all probability, CO_2.

In the peptone medium (fig. 4), the phenomena are somewhat different but the net results the same. Here, the unaerated medium (after a brief initial fall in pH) becomes slightly more alkaline while the aerated medium becomes much more alkaline. The growth curves and the pH curves in this figure present an almost mirror-like symmetry. Evidently the limitation of growth in this case is not due to acidity or alkalinity but to some other inhibitive waste products such as were postulated by Cohen and Clark (1919). Whatever these products may be they are evidently removed by aeration.

The phase of peak stability does not appear to be directly affected by aeration except so far as aeration has influenced the logarithmic phase. The phase of stability begins later and on a much higher level in the case of the aerated cultures. Once begun, however, it continues in both cases with little change for a period of fifteen to twenty-four hours at least. How much longer it would continue we did not find it practicable to determine.

The checking of logarithmic growth and the establishment of a phase of peak stability has been variously attributed to exhaustion of nutrients, to accumulation of inhibitory waste products and to some more obscure biological principle. The latter type of explanation is suggested by the work of Rogers and Whittier (1930) who found that stability develops even in a culture provided with fresh nutrient broth and with constant removal of waste products. Butterfield (1929) demonstrated a relation between total bacterial volume and the establishment of a stable population and Barnes (1931) failed to check growth by adding the waste products in filtrates of old cultures. In our case it seems that the establishment of peak stability at so much lower a level in the unaerated cultures can most probably be explained by inhibitory effects of toxic waste products or by decreased oxygenation, since there is no difference in the available food supply.

INFLUENCE OF SALT CONTENT UPON THE POPULATION CYCLE

Previous studies in this laboratory, as well as the work of other investigators, have made it clear that all cations in low concentration stimulate bacterial growth and in higher concentration inhibit it. The evidence along this line has been reviewed and discussed by Falk (1923) and in the recent contributions of Winslow and Dolloff (1928) and Winslow and Haywood (1931) with references to earlier publications.

Previous studies have, for the most part, been confined to the comparison of the number of bacteria present in media, with and without the presence of selected cations, after a given period of time. Complete growth curves have not before been determined in such a way as to present the trends of the population cycle in all its phases as affected by added cations.

In the present study, we have worked out complete growth curves for an aerated peptone medium in the presence of varying amounts of added NaCl ranging from a molarity of 0.1 to a molarity of 2.5. The results have been checked by observations of the effect of a single stimulating concentration and one or more toxic concentrations in unaerated peptone water, in aerated and unaerated lactose peptone broth and in aerated and unaerated Dolloff medium. We may first consider the reasonably complete data for aerated peptone water. They are presented in table 4 and in figure 6.

It will be noted that, in general, the influence of the added cation is exactly as observed by earlier workers. A 0.1 molar solution was found to be stimulating and solutions of 0.5 molar were found to be increasingly toxic by Hotchkiss (1923), Winslow and Falk (1923, a and b), Winslow and Dolloff (1928) and Winslow and Haywood (1931). The same phenomena are indicated by figure 6 and by the counts after four hours presented in table 4.

TABLE 4

Influence of various concentrations of NaCl on the growth cycle in aerated peptone water medium

Millions of bacteria per cubic centimeter

HOURS	SALT CONCENTRATION, MOLARITY						
	0	0.1	0.5	1.25	1.5	2.0	2.5
0	13	8	8	57	18	71	842
0.7		7	8				
1.0	13	7	8		11	19	
1.3		6	8				
1.5							465
1.7		5	5				
2.0	34	14	3	42	9	19	244
2.3		16	1				
2.7			1				
3.0	153	161	3		5		
3.3			10				
3.5							235
3.7		507	57				
4.0	430	699	143	23		18	
4.3		920	290				
4.5					5		
4.7		1,030	430				
5.0	836	1,160	489				145
5.3			621				
5.5		1,400					
5.7			697				
6.0	1,150	1,520	736	26		13	
7.0		1,680	903				
8.0	1,750	2,050	997			14	120
8.5				19			
15.0	2,170						
24.0	2,070			1			43
30.5							103
46.5							62

It will be noted of course that at molarities of 1.25, 2.0 and 2.5 we used high initial concentrations of bacteria. Figure 6, however, shows clearly that while the curve for 0.5 M NaCl rises and at its inflection is only slightly below that for the salt-free medium, the curve for 1.25 M drops steadily, that for 1.5 M more sharply and that for 2.0 M more sharply still. The curve for the 2.5 M concentration has a less steep slope but this is no doubt due to the enormous initial concentration of bacteria (842 million). We always find a mass effect caused by large numbers of living or dead cells which tends to neutralize any inhibitory action.

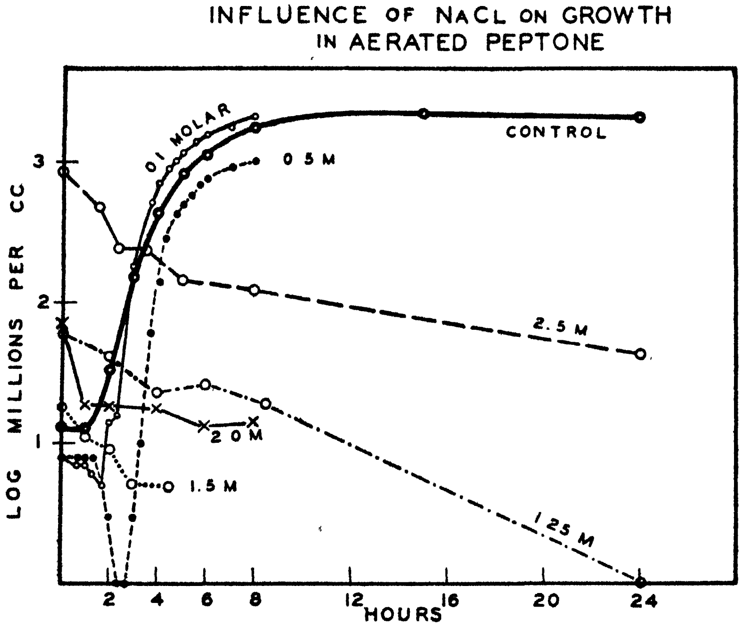

Fig. 6. Curves of Average Growth and Death in Aerated Peptone Water Containing Concentrations of 0, 0.1, 0.5, 1.25, 1.5, 2.0, and 2.5 Molar NaCl

New and interesting phenomena are revealed by the growth curves for the 0.1 M and 0.5 M salt concentrations. Here we note that the stimulating concentration (0.1 M) shows at first a distinct drop during the lag period; while the curve for the very slightly toxic concentration (0.5 M) shows a much more marked drop, followed by a subsequent rise, more abrupt than that of the salt-free medium. This gives us an entirely new conception of the effect of what we have called "stimulating" and "toxic" salt concentrations. It appears that the 0.1 M NaCl is first inhibitive and later stimulating, and that the 0.5 M NaCl is still more inhibitive at first and still more stimulating subsequently. The net effect after four hours is to give the result recorded in earlier studies from this laboratory,—for twenty-four- or forty-eight-hour observations,—a higher count in the 0.1 M NaCl and a lower count in the 0.5 NaCl than that recorded in the control. The actual rate of increase during the logarithmic phase is more rapid in the 0.1 M NaCl than in the salt-free medium (a generation time of twenty-one minutes[4] as compared with thirty-six minutes); but it is even greater in the 0.5 M NaCl (reaching the very low value of sixteen minutes[4]).

If the effect of "stimulating" salts is to increase permeability the greater dip in the lag period may be due to loss of CO_2 or other stimulating substances, the more rapid rise, to the removal of inhibiting waste products.

It will be noted that the toxic salts show (1) a less rapid decrease than the stimulating salt during the first two hours. Then follows (2) either a slight rise in the curve or at least an inflection showing a lessened rate of decline and finally (3) a steady decrease in numbers. If these toxic salts act by causing decreased permeability we might expect that lag would be slowed up, since the carbon dioxide or other stimulating substances would tend to be retained in the cells. The ultimate regular decline would be interpreted as due to the fact that reduced permeability prevents the elimination of waste products present within the cells in harmful proportions; and the intermediate period of temporary increase, or slackened decrease, might occur at the point when interference with excretion of beneficial products had permitted growth and before interference

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[4] The phase of most rapid logarithmic increase in the presence of these salt concentrations was of slightly shorter duration than in the plain peptone; the generation times are figured therefore on two-hour periods.

TABLE 5

Influence of 0.1 M NaCl on the growth cycle in various media, aerated and unaerated
Millions of bacteria per cubic centimeter

HOURS	PEPTONE WATER		LACTOSE-PEPTONE WATER		DOLLOFF MEDIUM	
	Aerated	Unaerated	Aerated	Unaerated	Aerated	Unaerated
0	8	11	14	13	6	4
0.3			15	12		
0.7	7		15	13		
1.0	7	10	13	13		
1.3	6		12	11		
1.7	5		26	31		
2.0	14	43	38	56		
2.3	16					
2.5				101		
3.0	161	106	296	146		
3.3			365	161		
3.7	507		382	172		
4.0	699	149	571	187		6
4 3	920		670	186		
4.7	1,030		733	265		
5.0	1,160	172	656	265		
5.5	1,400					
6	1,520		818	273	6	8
7	1,680	191				
8	2,050			275		
8.6						8
9.0			896	270		
10		200		267		10
11					6	14
12				250		22
13				252		
14						53
16						105
18						140
20						155
22						169
24		163	893	238	4	165
30					7	
33		245				
34						199
35			938			
36					4	
46						152
48					3	
49			576			

TABLE 5—*Concluded*

HOURS	PEPTONE WATER		LACTOSE-PEPTONE WATER		DOLLOFF MEDIUM	
	Aerated	Unaerated	Aerated	Unaerated	Aerated	Unaerated
58					2	
70					2	168
73			322			
76					4	
81					3	
107					237	
120			149			
131					284	

TABLE 6

Influence of toxic concentrations of NaCl on the growth cycle in various media, unaerated, and in the Dolloff medium, aerated

Millions of bacteria per cubic centimeter

HOURS	PEPTONE WATER—UNAERATED		LACTOSE-PEPTONE WATER—UNAERATED	DOLLOFF MEDIUM	
				Unaerated	Aerated
	2 0 м NaCl	2 5 м NaCl	2 5 м NaCl	1 5 м NaCl	1 5 м NaCl
0	290	835	537	204	145
1.0		387	415		
1.5			287		
2 0	109		192	102	
2.3		212			
2 7			273		
3.5		313	309		
4.0					76
5.0		613	198	53	
7 0			110		
8 0		415		46	
8.5					50
10	82		135		
12				44	39
22					15
24	25	226	72		
26				13	16
30		108			18
34			40		
35					6
47		15			
48					1
72			2		

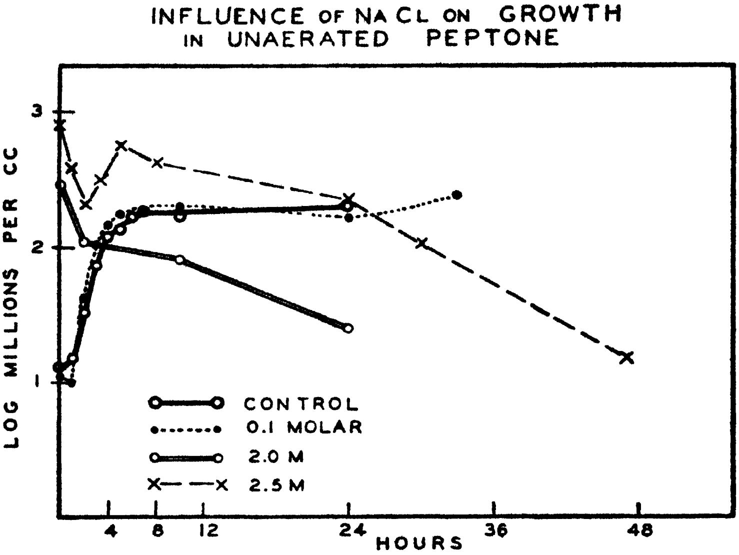

FIG. 7. CURVES OF AVERAGE GROWTH AND DEATH IN UNAERATED PEPTONE WATER CONTAINING CONCENTRATIONS OF 0, 0.1, 2.0 AND 2.5 M NaCl

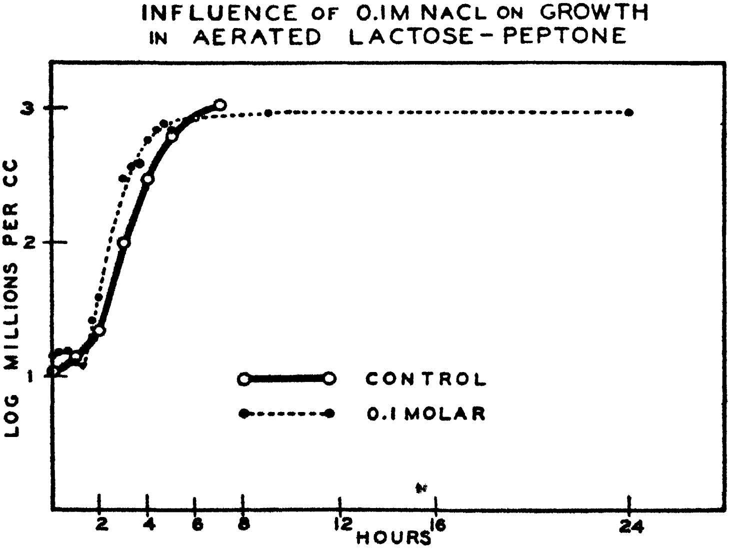

FIG. 8. CURVES OF AVERAGE GROWTH IN AERATED LACTOSE-PEPTONE WATER WITH AND WITHOUT 0.1 M NaCl

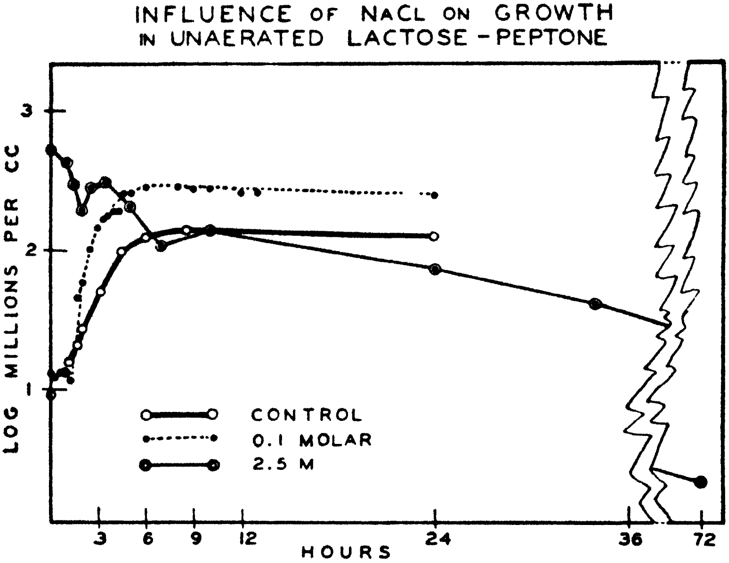

FIG. 9. CURVES OF AVERAGE GROWTH AND DEATH IN UNAERATED LACTOSE-PEPTONE WATER CONTAINING CONCENTRATIONS OF 0, 0.1 AND 2.5 M NaCl

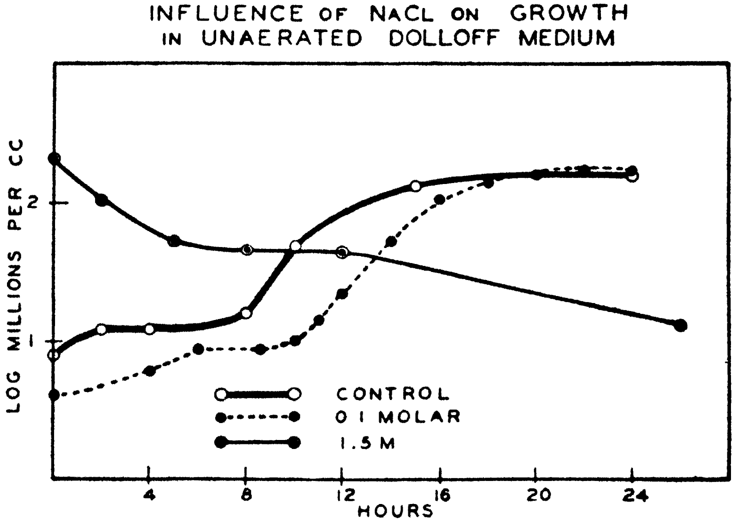

FIG. 10. CURVES OF AVERAGE GROWTH AND DEATH IN UNAERATED DOLLOFF MEDIUM CONTAINING CONCENTRATIONS OF 0, 0.1 AND 1.5 M NaCl

with excretion of such products in harmful concentration had checked it.

Our average data for stimulating salt concentrations in three media, both aerated and unaerated are presented in table 5; and results for toxic concentrations in unaerated peptone water, unaerated lactose peptone water and both aerated and unaerated Dolloff medium in table 6.

The data can best be interpreted by reference to figures 7 to 10 where the curves, with and without salt, are compared for each of the media, aerated or unaerated.

Figure 7 shows the results for the peptone medium without aeration. The general phenomena are identical with those which appear in figure 6 for the aerated peptone. The 0.1 M NaCl causes an initial decrease in numbers, followed by an increase slightly in excess of that of the salt-free medium. The generation time during the logarithmic phase is thirty-five minutes as compared with fifty-three minutes for the salt-free medium. The 2.0 and 2.5 M concentrations (starting with much higher initial counts) are highly toxic.

Figures 8 and 9 for the lactose-peptone water, aerated and unaerated, show similar results. In the aerated medium, 0.1 M NaCl reduced the logarithmic generation time from thirty-eight to thirty-one minutes;[5] in the unaerated medium it reduced it from fifty-three to thirty-one minutes.[6] The curves for the aerated Dolloff medium have not been plotted. It will be noted by reference to table 5 that in this medium with a stimulating salt concentration (0.1 M) the number of bacteria remained between 4 and 7 million for forty-eight hours, then dropped to 2 million and rose to 237 million at the one hundred and seventh hour. This was the only experiment with Dolloff medium prolonged for so great a period. Apparently, even in an aerated medium of such low nutrient value, the conditions for active growth may ultimately be established.

Figure 10, for the unaerated Dolloff medium, shows the usual phenomena, initially increased lag and later stimulation due to 0.1 M NaCl and marked toxicity of 1.5 M NaCl.

[5] Both calculated on a duration of logarithmic increase of three hours.

[6] Both calculated on a duration of logarithmic increase of two hours.

SUMMARY OF CONCLUSIONS

The present study throws light upon two problems.

In the first place, we find that aeration has definite and striking effects on the growth cycle of a bacterial population. It, first of all, prolongs the lag period for a short time in a rich nutrient medium and for many hours in a poor medium (Dolloff). This effect may be due to removal of CO_2 or to removal of intermediate products of cell metabolism necessary for growth stimulation. In a rich medium—once the lag period is passed,—the effect of aeration is to prolong the period of logarithmic increase and to accelerate the rate of logarithmic increase. The generation time may be reduced by at least 30 per cent by aeration and the final level of bacterial numbers at peak stability increased tenfold. This stimulating effect of aeration is presumably due to removal of toxic waste products of growth or to increased oxygenation. In lactose media the inhibitory waste products may be acidic in nature. In a sugar-free peptone medium they are certainly not. Yet the phenomenon is the same in both cases.

In the second place, these studies have given us a new conception of the influence of cations upon bacterial development. They have confirmed earlier work in showing that NaCl has a net stimulating effect in low concentration and a net toxic effect in higher concentration. They indicate, however, that both the mildly stimulating salt (0.1 M NaCl) and the mildly toxic salt (0.5 M NaCl) are toxic in the lag period and stimulating in the logarithmic period. The 0.5 M concentration is more toxic at first and more stimulating later than the 0.1 M concentration. It gives the lowest logarithmic period generation time recorded in any of our experiments. It would appear that salts (like aeration) do something to the bacterial cell which hinders it during the lag phase and stimulates it in the logarithmic phase. With very dilute salt (0.1 M NaCl) the initial decrease is slight and the subsequent stimulation considerable. With a less dilute salt (0.5 M NaCl) both initial decrease and later stimulation are greater but in the net result the decrease overbalances the increase.

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METABOLIC ACTIVITY OF THE BACTERIAL CELL AT VARIOUS PHASES OF THE POPULATION CYCLE[1, 2]

H. H. WALKER AND C.-E. A. WINSLOW

Department of Public Health, Yale School of Medicine

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EARLIER STUDIES OF BACTERIAL METABOLISM

Nitrogen metabolism. Studies of bacterial metabolism have, in general, centered about either nitrogen or carbon metabolism or the inter-relationship of these two processes. The general principles of nitrogenous metabolism in relation to cell structure, synthesis, katabolism, proteolysis and putrefaction, utilization of atmospheric nitrogen or ammonia and the like have recently been concisely and clearly reviewed by Rettger (1928). Some of the well established points of particular bearing on the present study are that an available source of nitrogen is the *sine qua non* of bacterial growth; that organic nitrogen compounds may also be utilized as sources of carbon and energy; that this utilization is accompanied by changes in the various non-protein nitrogen substances yielding a balance different from that which occurs when a carbohydrate is present; that sugar inhibits proteolysis by raising the acidity of the medium and in other ways as well; and that both utilization and production of ammonia are common properties of practically all bacteria.

The carbohydrate metabolism of bacteria has also been recently reviewed by Kendall (1928) in far more detail than could be here set forth. Historical aspects, structural carbon requirements, energy requirements, available compounds, preferential

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utilization of molecules of particular configuration, fermentation equations and products have been discussed.

The alteration of nitrogenous metabolism in the presence of fermentable sugar, early recognized from the attendant inhibition of indol production, and later reflected in the fact that toxin production by *C. diphtheriae* may be prevented by the presence of glucose, has been extensively investigated. Kendall and his coworkers over a span of twenty years have published one hundred articles on bacterial metabolism, many of which have dealt with this relationship and have emphasized the fact that utilizable carbohydrate protects protein from attack, or exhibits a "protein-sparing" phenomenon, which they have interpreted as indicating that the cells prefer carbohydrates to organic nitrogen as a source of energy.

Waksman (1917) suggested that the large accumulation of NH_3 in sugar-free media may be attributable to the wrecking for the sake of energy of more protein than can be utilized in cell structure. With added carbohydrate for energy, only the amount of nitrogenous matter needed for building protoplasm need be broken down.

Kendall (1922) elaborated a system of analytical methods whereby interchanges in the proportions of all nitrogenous groups —total, protein, non-protein, polypeptid, amino and ammonia nitrogen—could be followed, thus yielding a "nitrogen spectrum." This technic was applied to a number of organisms. Kendall and Bly (1922) found by its application to *Esch. coli* that unmistakable differences in ammonia content were shown in the presence or absence of sugar, while changes in amino nitrogen were insignificant. They regarded ammonia increase as "a quantitative measure of the intracellular utilization of protein for energy by bacteria."

Carbon metabolism. In studies of carbohydrate metabolism, a common procedure has been to inoculate a series of media containing different sugars with an organism and report on carbohydrate utilization in terms of development of acidity. This necessarily rough procedure requires little review other than to state that a need for caution has recently been made strikingly apparent by the work of Merrill (1930), who by simultaneous observation of sugar loss, CO_2 and NH_3 production, and reaction changes showed that apparently some organisms (13 strains of mycobacteria) when grown in sugar broth exhibited a carbohydrate metabolism in which no intermediate cleavage products were formed, CO_2 yield equalling or exceeding the maximum calculable from the sugar consumed,—utilization of carbohydrate appearing to have gone to completion with an alkaline reaction obtaining throughout.

Other workers have supplemented the somewhat elementary observation of reaction by quantitative determinations of various specific intermediary or final products of metabolism, such as acetic acid, lactic acid, pyruvic acid, formic acid and the like.

In still other metabolism studies particular attention has been directed to the gaseous products of life processes. This phase of activity has recently been summarized by Soule (1928).

Most of the early gas studies were made in a far from exactly quantitative manner. In extended reviews of the earlier literature, Keyes (1909) and Rogers, Clark and Davis (1914) pointed out that most of such work was done by means of the Smith fermentation tube, with some modification thereof to permit collection of the gases for analysis. Results were usually incomplete because of the fact that part of the CO_2 remained dissolved in the medium, part was lost by diffusion through the medium under the influence of a high CO_2 tension in the closed arm and a low tension in the open arm; while growth processes were necessarily uneven since the closed arm was anaerobic and the open arm partly aerobic. Nevertheless, useful and sometimes surprisingly significant results were sometimes obtained. A practical application of improved gas analysis methods was achieved in the distinction between high- and low-ratio groups of colon-aerogenes bacteria by Rogers, Clark and Davis.

A considerable gain in gas analysis technic came with the publication of Novy, Roehm and Soule's (1925) compensation manometer method. This provided the opportunity to follow a visible reflection of respiration changes with subsequent accurate burette analysis. Any type of medium could be used and any desired initial composition of gaseous environment provided. Actual respiratory quotients of $\frac{CO_2 \text{ produced}}{O^2 \text{ consumed}}$ could be calculated and, with suitable correction, made to correspond closely to the theoretical R.Q. for carbohydrate or protein media. The influence of anaerobic respiration could be detected, with O_2 taken from components of the medium, and CO_2 appearing secondarily from decarboxylation processes, the additional appearance of H_2 being registered by increased pressure. A summary of respiratory quotients for several bacterial and protozoal types has been included by Soule (1928) in his review. The values lay between 0.802 and 1.278. This apparatus also facilitated the study of the effect of increased and diminished O_2 tensions on growth and established the necessity of sufficient air supply for proper development of the tubercle bacillus. An aeration technic for removal of dissolved and combined CO_2 from the medium was employed at the close of manometric cultivation. This yield was absorbed in $Ba(OH)_2$ and determined by titration.

Other improved manometric methods have appeared of late. The micro-respirometer of Warburg (1926) used for studies on the metabolism of tumor cells has been recently employed in bacteriological applications by Eaton (1931) and by Burk and Lineweaver (1930). This method permits determination of respiratory rates in ten minutes of observation with such small volumes as 2 or 3 cc. of medium. It may be operated with a normal atmosphere or the apparatus may be filled with a desired test gas. When operated with an alkaline absorbent present the falling pressure responds to O_2 consumption; without an absorbent, it reflects the difference between O_2 consumed and CO_2 released.

This micro-respirometer was applied by Eaton to determination of the respiration of actively growing staphylococcus cultures lysed by bacteriophage and it revealed a rate of O_2 consumption greater than could be accounted for by the number of intact cells present. Moreover, lysed cultures with negligible numbers of living cells present continued to absorb O_2 and release CO_2 for several hours. Eaton concluded that either bacteriophage itself or some product of its action on, or in combination with, bacteria respired. This work was of particular interest to us in that cell numbers were determined at frequent enough intervals to yield a growth curve and both the gas observations and the addition of the bacteriophage were timed to fit within chosen points of a growth period.

Burk and Lineweaver (1930) used the Warburg micro-respirometer for study of the influence of fixed nitrogen, O_2 pressure and other factors on the respiration rate and growth rate of Azotobacter. The interesting observation was made that, since increases in the number of organisms accompanied temporal increases in the rate of O_2 consumption, it was possible by observation of O_2 consumption rates for a few hours to determine qualitatively and semi-quantitatively the amount of growth and the rate of nitrogen fixation.

A simple respirometer, in which CO_2 produced by yeast was measured by pressure changes in a fixed gaseous volume, has been recently described by Rahn (1929a, 1929b) and called "The Fermentometer." By making frequent time observations, time-number-product relationships were established which permitted formulation of fermentation constants. The author concluded that the decrease in rate observed in alcoholic or lactic fermentations was proportional to the percentage of total possible products already formed.

Another recent study of metabolism has approached the problem differently. Bayne-Jones and Rhees (1929, a and b) employed a micro-calorimeter to measure the rate of heat production. Here growth curves were also established, and bacterial numbers noted for the chosen time intervals.

In the work of Eaton, Burk and Lineweaver, and Rahn O_2 consumption or CO_2 production has been related to cell growth. No investigator, so far as we are aware, has made a systematic quantitative study of the end products of simultaneous nitrogen and carbon metabolism in relation to cell population and at various known phases of the population cycle. This was the objective of the present investigation.

TECHNIC OF PRESENT INVESTIGATION

General plan. The present studies have been conducted with a single strain of *Esch. coli*, cultivated in three different media, peptone water, (1 per cent peptone) lactose-peptone water (0.5 per cent peptone and 0.5 per cent lactose) and Dolloff medium (0.5 per cent ammonium tartrate, 0.5 per cent lactose and 0.002 per cent di-ammonium phosphate). A stimulating solution of NaCl (0.1 M) was added to the peptone medium in certain experiments and a toxic solution of NaCl (1.5 M) was added to the Dolloff medium in certain other experiments. Meat extract was omitted from the media in order to avoid compounds of uncertain composition and to keep all three media mutually comparable.

All cultures were made in a medium continuously aerated with a stream of air which had been freed from NH_3 and CO_2. The apparatus has been fully described by one of us (Walker (1932)) in a preceding paper. This procedure gives a much more normal population than does the usual bacteriological technic in which the organisms are quickly smothered by their own waste products. Thus, Novy, Roehm and Soule (1925) found that *Esch. coli* cultivated on an agar slant in a closed manometric system in two days reduced the O_2 content to 2 per cent and increased the CO_2 content to nearly 16 per cent. Magoon and Brunstetter (1930) have shown the value of aeration and of a continuous circulation of medium in promoting more normal growth.

The growth curves of the organism were carefully determined for each condition as described in an earlier communication (Winslow, Walker and Sutermeister (1932)). Determinations of metabolic products were made for time intervals corresponding to the lag phase, the phase of logarithmic increase and the phase of peak stability and (in the case of Dolloff medium plus toxic salt) to the phase of logarithmic decrease. The way in which these time intervals fitted into the curves for the population cycle is illustrated in figure 1. An uninoculated control was run in parallel with each culture, similarly aerated, and subjected simultaneously to all chemical and bacteriological tests. All yields were taken as net excess of the culture over its parallel control.

Bacterial counts, pH readings, and determinations of CO_2 and NH_3—N content of the medium were made at the beginning and end of each time interval. To the increase of these gases in the medium from start to close of each period were added the amounts of CO_2 and NH_3—N carried off from the culture bottles by the aerating current during the period, as collected and determined by the absorption train.

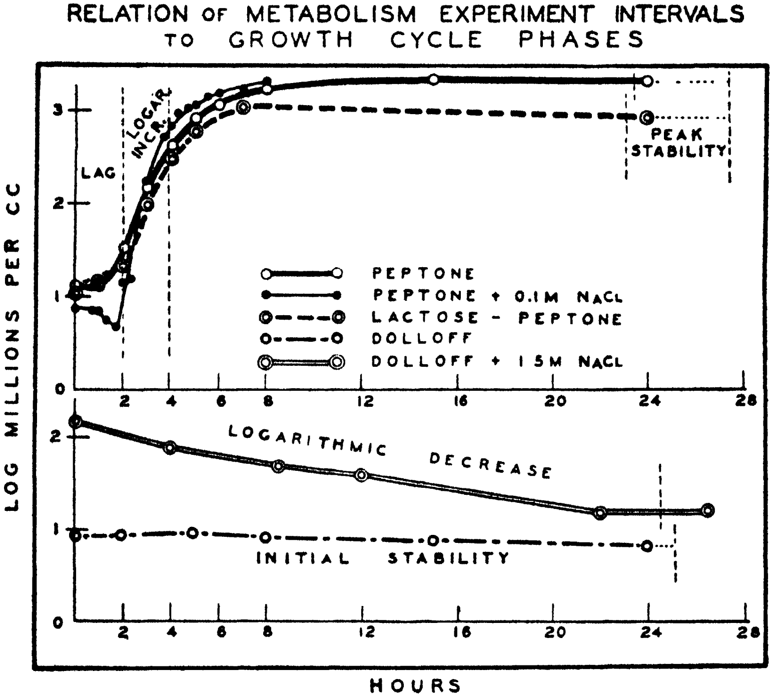

FIG. 1. RELATION OF PERIODS SELECTED FOR OBSERVATION OF METABOLIC ACTIVITY TO AVERAGE CURVES OF AERATED GROWTH OR DEATH IN THE VARIOUS MEDIA

Bacteriological methods. At the beginning and end of each time interval, the number of viable bacteria present was determined by plating in triplicate on nutrient extract agar at 37°, since for the purposes of the investigation it was essential to estimate the number of cells active during a given period of growth so that a value indicative of metabolic activity per individual cell could be calculated. The details of the preparation of the culture media and of the bacterial suspensions used for inoculation have been described by Winslow, Walker and Sutermeister (1932).

The rate of production of CO_2 or NH_3—N per bacterium per hour in milligrams $\times 10^{-11}$ has been calculated for the stable period of saturated population by means of formula (1). During the course of such metabolic studies, which were conducted from about the twenty-fourth to the twenty-eighth hour with cultures which had been grown under the influence of continuous aeration, relatively little increase or decrease occurred, as shown by initial and terminal counts. It was therefore assumed that a fair approximation of the number of viable cells active during the four-hour period would be represented by the arithmetical mean between the plate counts at start and close of the period. Time was of course included in the denominator.

$$(1) \qquad p = \frac{P}{\frac{b + B}{2} \cdot t} = \frac{2\,P}{(b + B)\,t}$$

where p = amount of product per bacterium per hour.
P = total product during time t.
t = time of gas collection in hours.
B = number of bacteria at start of time t.
b = number of bacteria at end of time t.

Calculation of the rate of production during the logarithmic period is a very different matter, for the number of bacteria is continually increasing. A formula whereby the total increment of substance yielded during this phase could be distributed as an average rate among the producing cells was introduced by Buchanan (1918) and was based on an integration by means of the calculus. Inasmuch as typographical errors occurred in the original publication, the formula (2) will be used as set forth in a paper by Bayne-Jones and Rhees (1929b), except that the symbols have here been changed and the modulus transferred to the denominator, while the log quotient in the numerator has been written as the difference of the logarithms. Both forms of writing the formulation are presented in order that no confusion may result if it is desired to trace the derivation supplied by Buchanan in Bayne-Jones and Rhees' publication.

$$(2) \qquad p = \frac{P\,(\log b - \log B)}{0.434\,(b - B)\,t} \quad \text{or} \quad M = \frac{S\ 2.303 \log \frac{b}{B}}{t\,(b - B)}$$

where p = amount of product per bacterium per unit time = M.
P = increment of product in time t = S.
t = duration of production in hours.
b = number of bacteria at end of time t.
B = number of bacteria at start of time t.
$\frac{1}{0.434}$ = modulus of common logarithms = 2.303.

This formulation is mathematically a close approximation to truth if its assumptions be met. The danger of its use, however, lies in the fact that it assumes that the cells are increasing at a constant rate by pure geometrical progression and that the product is being excreted by each individual cell at a constant rate during the period. The assumption that any culture ever actually achieves constant geometrical increase is probably liable to challenge but the approximation to logarithmic growth is undoubtedly close enough to avoid serious error. The greater danger lies in assumption of constant rate of cell excretion during time t.

Bayne-Jones and Rhees' application of the formula to calculation of rate of heat production per cell per hour, in which they used total heat cumulatively from inoculation to each successive hour, with similar treatment of bacterial increases and with the inclusion of periods which their growth curves showed to be characterized by lag or negative growth acceleration instead of logarithmic increase, has been criticized by Wetzel (1929), who claimed that the data of Bayne-Jones and Rhees revealed a continually changing rate of production per cell which rendered any use of this formula for their data theoretically untenable. Rahn (1930) concurred in objection to the use of the formula for cumulative products, time and cells instead of for increments during short periods; he recalculated Bayne-Jones and Rhees' data by successive periods instead of by cumulative ones from time 0 and the results obtained showed even more striking differences in the rates for individual hours, which diminished from 15.3×10^{-9} calories per cell during the second hour to 1.4×10^{-9} for the fourth hour, though both hours were apparently in the logarithmic period. He reasserted that the formula was accurate if properly applied and if the time intervals chosen were "short enough;" and finally concluded that Wetzel's objection was sustained only if the formula were applied to uses for which it was not intended.

Hot upon the heels of the above discussion, we have made bold to use the same formula in this paper! But we have done so with restrictions in use and with reservations, in interpretation. After very careful establishment of aerated growth curves for all our media, we chose for study the short span of two hours, the third and the fourth in the culture's history, which appeared to be most definitely of logarithmic nature. We have used in calculation only the increments of time, bacteria and gases produced over that period. We have therefore been able to compute comparative rates of gas production in peptone, lactose-peptone and peptone + 0.1 M NaCl which are based on similar culture age, similar population phases, similar duration of time, essentially similar bacterial numbers, approximately similar magnitudes of total product and finally closely similar *k*'s of growth increase. Moreover, the rates thus computed for individual experiments in a single medium show a remarkable agreement with one another, which tends to indicate that the computed rate is functioning as an index of some fairly constant quantitative activity. Therefore, when it is stated that *Esch. coli* organisms in 1 per cent peptone at 37°C. produce CO_2 at the rate of 52×10^{-11} mgm. per cell per hour during logarithmic growth occurring between the second and fourth hour of the population's age, the reader will please not assume the writers to advance this figure in the belief that it holds as a true rate for every bacterium for every five minutes during the two hours. It does however, in our opinion, represent an interesting and useful characterization of rates during the period and is, in some degree at least, a smoothed or mean reflection of the degree of metabolic activity occurring therein.

The computation of active cell population during the lag period is subject to insuperable difficulties. The period actually observed (the first two hours of incubation) included often (perhaps always) an initial drop in numbers, a period of true lag and a period of accelerating rate of increase leading up to logarithmic multiplication. No mathematical formula for rate of metabolic activity can be practically derived which will describe this situation with accuracy. We have therefore only attempted for this period to state maximum and minimum rates, computed by dividing the total product P first by $B.t$ and then by $b.t$. The first procedure (dividing by time and by the initial number of bacteria) gives an average rate of cell metabolism which is too high, since for the greater part of the period more cells were present than allowed for. The second procedure (dividing by time and by the final number) gives a rate of cell metabolism which is too low, since less cells were present during most of the period than the result would assume. In our tables we give these two figures with the assumption that the actual mean rate lay somewhere between the two limits and that the rate for various subdivisions of the period must have varied widely. This indefinite zone within whose limits the mean rate probably lay has been depicted in figure 2 by the dotted portion above the solid blocks of the graph, the height of the latter of course representing the minimum rate.

Hydrogen ion concentration. The reaction of the medium was determined at the start and close of each interval by means of the quinhydrone electrode, using portions of about 2 cc. withdrawn when culture samples were taken for plating. This method was frequently checked by colorimetric determinations, using LaMotte standard indicators and color standards in a comparator block to offset color and turbidity effects.

The reference electrode solution of M/20 potassium acid phthalate was made at two-week intervals by dilution from a M/5 stock solution which had been prepared by careful weighing of c.p. salt on analytical balances and checked to within the limits of experimental error by titration with standard NaOH and phenolphthalein. The Eppley standard cell on which all E.M.F. readings ultimately depended was twice compared with another standard cell during the experimental period, with observed discrepancies of only 0.0006 and 0.0004 volt between the two cells. Since this possible error represented less than 0.01 pH interval the check was considered satisfactory. Readings were made with a Leeds and Northrop enclosed lamp and scale galvanometer by means of a L. and N. "simplified type" potentiometer, graduated in intervals of 0.0005 volt. Electrode vessels and test tubes used for collection of samples were of Pyrex glass and were cleaned in potassium dichromate-sulphuric acid solution followed by careful tap and distilled water rinsings.

Results with the electrometric method were highly satisfactory over a long period, barring a few short intervals when electrode troubles were encountered. Readings on a given medium were repeated at yearly intervals within a variation of a few hundredths of a pH unit. Tests against known buffers lay within a 0.05 pH variation. Since the second decimal figures have been rounded off, it is likely that most values as listed were correct within ±0.1 pH.

The colorimetric tests served to indicate that the electrometric outfit was in perfect working order, and they of course replaced the electrometric tests for the most alkaline solutions. Some of the colorimetric tests were applied to media diluted to reduce color and turbidity and lessen the size of samples withdrawn from the experiments. In the light of work recently done by Fawcett and Acree (1929) the dilution here employed, which never exceeded 1:10 and was usually 1:5 or 2:5 probably did not influence results appreciably.

Ammonia nitrogen. It was recognized that ammonia nitrogen might represent a resultant of both anabolic and katabolic activity. Yet it seemed likely to be significant as a measure of nitrogenous metabolism in a broad sense (including both extracellular and intracellular activities).

Since *Esch. coli* is not an active ammonia producer, the yields to be expected were small and rather delicate technics were involved.

The air with which the culture was aerated was freed from ammonia by passing it through a Milligan spiral gas-washing bottle containing strong H_2SO_4 (see Walker (1930, 1932)). The air after it had been drawn through the culture was passed through an 8- by 1½-inch cylinder with Folin ammonia absorption bell, containing 100 cc. of 0.05 normal H_2SO_4 for absorption of the entrained NH_3. The NH_3–N was determined at the end of the period by neutralization and direct Nesslerization of an aliquot portion, according to the Standard Methods of Water Analysis (1925) of the American Public Health Association but without preliminary clarification. The reasons for using an acid solution of the strength stated are discussed in an earlier communication (Walker (1932)) in which it is also demonstrated that retention of ammonia was essentially complete. Tests for false-positive results due to entrainment of culture medium showed that this factor was appreciable only in the Dolloff medium, (which contained 76.5 mgm. of NH_3–N per 100 cc.). No serious error was usually introduced here, however, since all results are expressed in terms of the excess of yield from an inoculated culture over that from a sterile control. In peptone and lactose-peptone media the amount of NH_3–N found in the air which had passed through the sterile control varied between 0.002 and 0.005 mgm. and our results are expressed only to the nearest hundredth of a milligram. With Dolloff medium, on the other hand, the air from the control flask contained 0.04 to 0.13 mgm.

Dissolved or combined NH_3–N remaining in the medium itself was determined by the Van Slyke and Cullen (1914) modification of Folin's aeration method, with some further modification, as follows. In the Dolloff's medium and in peptone cultures one day old it was possible to employ the published method, the only change lying in the selection of strengths of absorbing acid adapted to the range of yields anticipated. Under all other conditions, it was found necessary to replace the standard acid absorbent by 0.05 N H_2SO_4 and to determine the NH_3–N by direct Nesslerization as had been done in the case of the aerated ammonia yields (see Walker (1932)).

The problem encountered in our medium analyses may be made clear by a specific illustration. Certain trial metabolism studies indicated that in some parts of the work media analysis would have to be applied to a content of either NH_3-N or CO_2 below 4 mgm. per 100 cc. This small amount could not be discarded as inconsequential, since it might at times represent from one-quarter to three-quarters or more of the entire yield. Moreover, in either the NH_3 or the CO_2 analysis large samples could not be handled and small aliquots had to be used. For example, at the concentration of 4 mgm. per cent, the actual 5 cc. portion of culture medium submitted to ammonia analysis would contain only 0.2 mgm. of NH_3–N, while a 1 cc. aliquot for CO_2 analysis would contain only 0.04 mgm. of CO_2. In the case of ammonia, if absorption in the ordinary N/50 acid was attempted, the entire yield represented by the difference between original absorbent and remaining acid titrated would be only 0.71 cc. With measurement of two volumes of standard solutions, involving four burette readings, required for each determination by the usual physiological method, the possibility of accuracy in arriving at such a figure appears rather slight. If now it was desired to distinguish between a yield of 4 mgm. per cent in one medium and 2 mgm. per cent in another, the problem would be very difficult, with an experimental error perhaps 50 to 100 per cent of the yield. On the other hand, if recourse were had to markedly more dilute standard solutions, as perhaps N/500, in order to place the titration differences in a range of volumes which could be more accurately observed, other errors would begin to appear, in the dilution of the standard solutions, in their preservation and in the much less sharp endpoints of indicators. In preference to the above methods, it was decided to rely for such small amounts on Nesslerization, since a yield of the content cited, 0.2 mgm. from the aliquot, equals ten to twenty times the range in which color standards are most sensitively distinguishable.

It was supposed that analysis for NH_3–N in media would be more reliable if the bacterial cells were removed before testing. This point was investigated by arranging to compare yields from plain media with those from similar media containing added bacterial cells or from which added cells had been at once removed by centrifugation. The results showed NH_3–N of media plus organisms exceeding that of sterile media in seven out of eight tests and equal once. The centrifugated supernatants also exceeded the sterile media—though usually to a lesser degree—in six tests, with the seventh one showing equality. Six out of nine times, the cell-containing media exceeded the centrifugated media and three times results were equal. Since the method was subject to some variation and the cell-containing media were always greater or equal, but never less than, the other media in determined NH_3–N, it was concluded that the bulk of evidence favored centrifugation.

The policy followed in all metabolism experiments was, therefore, to remove to capped centrifuge tubes at the start and close of each metabolic period about 9 cc. of culture and of control medium. The centrifuge was started at once and allowed to run while plating and other analytical processes took place. This of course involved some loss of time before the samples could be analyzed and meant that the values obtained were probably slightly higher than amounts present at the sampling time. It became possible, however, to systematize routine so that the intervals by which each ammonia test lagged behind the period boundaries varied little from day to day. Moreover, the cooling of the samples from 37°C. to room temperature probably retarded metabolism and tended to reduce false positive errors. The principle of including centrifugation in the technic was further sustained by running many of the medium analyses, during the metabolism experiments, simultaneously, with and without centrifugation.

Five cubic centimeters from the supernatant constituted the aliquot for analysis. One drop of caprylic alcohol was added to the specimen but not to the absorbent tubes. Slow, pressure aeration for ten minutes was followed by rapid vacuum aeration for thirty minutes.

Error in our NH_3—N determinations due to matching of the Nessler color tubes was, we believe, not over 10 per cent. In most experiments the bulk of the total ammonia yield came from the medium component rather than from the aeration harvest and the accuracy of the results was indicated by a compilation of

64 analyses of sterile control media. In 16 analyses of peptone medium there was found a mean content of 0.95 mgm. per 100 cc. with a mean error of 0.07 mgm. or 7.4 per cent; in 12 analyses of peptone plus 0.1 M NaCl, the mean content was 0.88 mgm. with a mean error of 0.05 mgm. or 5.7 per cent; in 19 analyses of lactose-peptone, the mean content was 0.49 mgm. and the mean error 0.05 mgm. or 10.2 per cent; in 8 analyses of Dolloff medium plus 1.5 M NaCl,[3] the mean recovery was 75.74 mgm. of a theoretical 76.51, and the mean error, 0.86 mgm. or 1.1 per cent; in 9 analyses of Dolloff medium,[3] the mean content found 75.17 mgm. and the mean error, 1.33 mgm. or 1.8 per cent.

Thus, we believe that results in the metabolism experiments, stated to the nearest hundredth of a milligram, are essentially correct and significant, with the single exception of the Dolloff medium. Here, the large amount of NH_3—N originally present brought the slight changes produced by the bacteria within the range of even a very low experimental error observed in that medium; and no reliable NH_3—N data can be given for it.

The significance of some absolutely small results may be indicated by citing the determination of NH_3—N in the air passed through the peptone medium during the phase of peak stability. In this instance 87 per cent of the total NH_3—N formed was collected from the air drawn through the culture. The amount determined was only 2.1 mgm. per 100 cc. of the producing culture but this was 700 times the amount present in the air drawn through a sterile peptone control.

Carbon dioxide. The CO_2 carried off through the aeration train was absorbed in a 250 cc. Erlenmeyer flask with a Brady-Meyer absorption tube containing 75 cc. standard N/10 $Ba(OH)_2$. A Bowen potash bulb on the distal side controlled the completeness of absorption. The collected CO_2 was determined by difference by titration of residual $Ba(OH)_2$ with standard HCl using thymolphthalein as an indicator (see Walker (1932) for details of technic and reasons therefor).

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[3] The yields from Dolloff medium, with or without NaCl, were determined by titration in the usual Van Slyke and Cullen manner, instead of by Nesslerization, since the ammonia content was high.

The determination of CO_2 in the medium presented greater difficulties. Considerable preliminary work showed that acidulation of aliquot portions of the medium, aeration with CO_2-free air and absorption with $Ba(OH)_2$ could not be relied upon. With such very small quantities, an alkali strong enough for effective absorption would return the yield in terms of too small a volume for reliable titration, while a more dilute alkali, although favoring accurate burette reading, would allow part of the CO_2 to pass unabsorbed as we readily demonstrated. Serial absorbents as employed by Novy, Roehm and Soule (1925) for this type of medium analysis were utterly out of the question for such small amounts.

The problem of CO_2 determination in our media was finally solved by use of the modified, fine-bore ("precision type") blood gas apparatus described by Van Slyke and Stadie (1921). This was equipped with a mechanical shaker operated by electric motor. The stem was carefully water-calibrated twice and readings corrected by the resulting calibration curve. Inasmuch as considerable work was to be done on solutions containing one-tenth to one-fiftieth or less of the CO_2 normally encountered in the blood analyses for which the machine was devised, many tests were conducted with graded small amounts of known solutions in order to establish the degree of accuracy obtainable in various ranges of yield.

Manipulations and reagents were as described by Van Slyke and Stadie. Analyses were performed in aliquot portions of 1 cc. measured with a pipette graduated between two marks and the contents were delivered under distilled water in the receiving cup. Normal lactic acid was used for acidulation. Gas volumes were read at atmospheric pressure over the acid solution, with use of the published correction factors for dissolved and reabsorbed gas content. No water jacket was employed, since its stopper would have interfered with the reading of small volumes and observed temperature variations were not such as to require its use. The method of magnification of small volumes by pressure reduction of 500 mm. of mercury, as described by Van Slyke and Stadie, was carefully tested, but with our apparatus appeared to offer no greater accuracy. Barometer readings were taken at the time of each pair of determinations. Gas volumes extracted from the blank control media were deducted from those yielded simultaneously by the culture, and the excess of the latter as cubic centimeters of CO_2 were corrected by the Van Slyke and Stadie factors, reduced to 0° and 760 mm. and converted to milligrams per 100 cc.

As with ammonia, the accuracy of determining total carbon dioxide produced depends on the relative errors of aerated and media content yields.

The principal error in the aeration yield was probably only that involved in titration, since the saturated $Ba(OH)_2$ controls indicated 100 per cent efficiency of absorption by the standard solution in the Brady-Meyer tubes. The inaccuracy, even when the amount of CO_2 was approximately equal to only 5 cc. of N/10 $Ba(OH)_2$ was not over 5 to 10 per cent of the recorded yield.

The accuracy of medium analyses for dissolved and combined CO_2, as made by the fine-bore Van Slyke machine, was investigated by trials in which several dilutions of a standard Na_2CO_3 solution in distilled water and in the various culture media were analyzed. The culture media were included in these tests in order to see whether they might either inhibit the recovery of known amounts or might add false positive values thereto, neither of which turned out to be the case. The results of fifty-five test analyses have been reviewed and show that with added CO_2 of 5 mgm. per cent or over, mean recoveries checked always within 9 per cent. With only 2 mgm. per cent added, four series of tests showed mean errors of 3, 3, 3 and 13 per cent respectively. Three test series made with added concentrations of CO_2 of 1 mgm. per cent, 0.5 per cent and 0.4 per cent showed errors of 12, 5 and 22 per cent respectively. Altogether, down through concentrations of 2 mgm. per cent a mean recovery of better than 90 per cent was achieved in all but one of fourteen groups of tests. Since analysis was always performed on aliquots of 1 cc., this means that in the case of the 2 mgm. per cent solutions measurements were actually made of only 0.02 mgm. of CO_2, during which the mean loss was found to have been less than 0.002 mgm., which was obviously a satisfactory performance for the machine. The recoveries with even lower concentrations were good but would probably be considerably less consistent.

It appears reasonable to assume that media contents of over 1 mgm. per cent CO_2 may have been determined with a mean loss of not over 10 per cent. Since the CO_2 medium content at the start of each metabolic period was deducted from the terminal value the possible experimental errors in one direction were somewhat increased by the subtraction involved.

Sugar content of medium. The reducing sugar content of media containing lactose was determined at the start and close of each metabolic period. The micro-reagent method of Shaffer and Hartmann (1920–1921), as adapted to culture media by Stiles, Peterson and Fred (1926), was employed. Strict adherence to the fifteen-minute heating period and to uniform cooling followed by prompt titration were practiced. The reagents and standard solutions were prepared from Baker's analyzed c.p. quality chemicals. Each determination involved three tubes, containing respectively the culture sample, sterile control medium sample and reagent blank. The clarification method employing basic lead subacetate (Horne reagent) was applied both to culture and control media. Ten cubic centimeter test portions were diluted to 50 cc. in volumetric flasks and aliquots of 2 cc. from the settled supernatant were used for analysis. A high degree of uniformity was obtained, the mean variation with sterile control lactose-peptone, Dolloff medium and Dolloff medium plus salt being respectively 2, 7 and 5 per cent of the mean yield in a total series of 33 determinations.

The irregularities which Magee and Smith (1930) have recently claimed must be tolerated when the Shaffer-Hartman micro method is applied to sugar broth media apparently have been somewhat minimized in our media, which contained no meat extract or meat infusion. Nevertheless, the actual change in concentration of sugar over so short a period as two to four hours was apparently too small in amount to register clearly over even so low a mean variation as we observed in the control media. The difficulty is not so much that the method was erratic but that the magnitudes of yield sought were apparently too low for this or any other method of which we are aware. Hence, we have omitted discussion of sugar consumption results from the present communication.

RESULTS

Scope of experiments. Altogether, 39 different experiments are here reported. They include ten different conditions as follows: lag period, period of logarithmic increase and period of peak stability in the peptone medium; the lag phase and the period of logarithmic increase in peptone plus a stimulating salt, 0.1 M NaCl; the lag phase, phase of logarithmic increase and phase of peak stability in the lactose-peptone medium; a phase of initial long-continued stability in Dolloff medium; and a phase of decrease in Dolloff medium plus a toxic salt, 1.5 M NaCl. Four check experiments were made under each of these conditions except in the Dolloff medium plus 1.5 NaCl, which included only 3 experiments.

The full protocol for a typical series of experiments is given in table 1. In this table, the vertical columns represent individual experiments with the exception of the right-hand column, which is their average. Exceptions to the method of averaging were made in the case of computed values, such as ratios and constants, which were recalculated from their mean components. Rates per cell per hour, however, were, because of their common base, averaged directly. The net result of these processes is that an hypothetical "average culture" has been set up which may be discussed with greater simplicity and economy of words than could the individual cultures. This procedure seems justified in view of the general concordance of results. Therefore in later tables we shall present only the averages of the 3 to 4 similar experiments conducted under identical conditions.

The horizontal lines in table 1 are largely self-explanatory. They cover explicitly the time features of the period studied, the population changes and growth characteristics, the changes in medium reaction, and the production of both CO_2 and NH_3 nitrogen. For these gases there are given the yields in milligrams collected by the aeration current ("aerated") and those deter-

TABLE 1

Protocol of metabolism experiments nos. 51–54

Phase: Logarithmic increase. Medium: One per cent peptone. Culture volume: 100 cc.

	INDIVIDUAL EXPERIMENTS				AVERAGE
	No. 51	No. 52	No. 53	No. 54	
Period:					
Hours from inoculation	2–4	2¼–4¼	2¼–4	2¼–4	2¼–4
Duration, minutes	122	123	109	110	116
Population × 10^9:					
Initial (B)	2.11	2.43	4.73	4.25	3.38
Terminal (b)	46.30	48.30	42.30	34.20	42.78
Increase (b-B)	44.19	45.87	37.57	29.95	39.40
Ratio increase (b/B)	21.9	19.8	8.9	8.0	12.7
Velocity coefficient, K (hours)	1.52	1.46	1.21	1.14	1.31
Generation time (minutes)	27.4	28.5	34.5	36.5	31.6
Type colonies, S/R:					
Initial	1.7	1.4	1.1	0.6	1.2
Terminal	2.3	1.3	1.3	0.7	1.4
Reaction, pH:					
Initial	6.6	6.5	6.6	6.6	6.6
Terminal	6.8	6.9	7.1	7.0	7.0
CO_2 produced, mgm.:					
Aerated	12.89	13.72	13.66	11.28	12.89
Residual	2.28	1.85	3.16	2.07	2.34
Total	15.17	15.57	16.82	13.35	15.23
Equivalent millimols	0.344	0.353	0.382	0.303	0.346
NH_3-N produced, mgm.:					
Aerated	0.00	0.00	0.00	0.00	0.00
Residual	1.00	1.60	1.60	1.18	1.35
Total	1.00	1.60	1.60	1.18	1.35
Equivalent millimols	0.071	0.114	0.114	0.084	0.096
Ratio CO_2/NH_3-N:					
Milligrams	15.2	9.7	10.5	11.3	11.3
Millimols	4.8	3.1	3.3	3.6	3.6
Rate/cell/hour (mgm. × 10^{-11})					
CO_2	52.2	49.5	54.0	50.8	51.6
NH_3-N	3.4	5.1	5.1	4.5	4.5

mined by increase in medium content ("residue") and the sum of both, i.e., the total yields. This last figure is also expressed as the equivalent number of millimols. Next to the last pair of horizontal lines shows the ratio of $CO_2 \div NH_3$–N both by weight (milligrams) and by molecules (millimols). The last pair of lines give the computed rates of production of the two gases during the period in terms of milligrams $\times 10^{-11}$ per bacterial cell per hour. All quantitative figures have been proportioned to a base of 100 cc. of culture volume.

In connection with the expression of growth characteristics there were calculated for the two-hour logarithmic period experiments the generation time in minutes (g) and the velocity coefficient of increase per hour (k). These have been computed according to well-known formulae.[4]

The type colony ratio S/R was obtained by counting on the triplicate plates the number of surface colonies which appeared to be definitely of smooth or rough appearance.

Peptone medium. We may consider first the results in the 1 per cent peptone medium which are summarized in table 2.

It will be noted that during the first two hours in the plain peptone water (column 1) the reaction of the medium became slightly more acid and the number of bacteria somewhat more than doubled. Rather large yields of CO_2 were produced (over one-third remaining in the medium) with a relatively small

[4]
$$g = \frac{t \log 2}{\log b - \log B}$$

and

$$k = \frac{ln\ 2}{g^1} = \frac{log\ 2}{M \cdot g^1}$$

where g = generation time in minutes.
t = duration of period in minutes.
b = bacteria at close of time t.
B = bacteria at start of time t.
k = velocity coefficient of increase per hour.
g^1 = generation time in hours.
m = modulus of common logarithms, value 0.434.
log = common logarithm to base 10.
ln = natural logarithm to base e.

Detailed derivation of these formulae, the basis of which was attributed to Buchner, Longard and Riedlin (1887) may be found conveniently assembled in Buchanan and Fulmer's (1928) text.

quantity of NH$_3$–N, none of which passed off through the aeration train. The amounts of these two products were, however, nearly equal when expressed in millimols, suggesting that CO$_2$ and NH$_3$ were produced approximately molecule for molecule. The production per cell cannot be determined accurately on account of

TABLE 2

Average results in peptone medium with and without stimulating salt

	PHASE OF				
	LAG		Logarithmic increase		Peak stability
	Peptone	Peptone + 0 1 M NaCl	Peptone	Peptone + 0 1 M NaCl	Peptone
	(1)	(2)	(3)	(4)	(5)
Period studied, hours after original inoculation	0–2.2	0–2.0	2.2–4.0	2.0–4.0	23.5–27.5
Bacterial population at start of period × 10^9	1 45	0 95	3.38	2.70	253
Change in bacterial population (*b*/*B*)	2 3	2.9	12.7	30 9	1.3
Reaction, pH:					
Initial	6.8	6 8	6.6	6 5	8 3
Terminal	6.6	6 5	7.0	7.1	8.4
CO$_2$ produced, mgm.:					
In air	2.26	2.20	12.29	15.06	18.48
In medium	0.89	1 35	2.34	2.88	−3.27
Total	3 15	3.55	15 23	17.94	15.21
Total, equivalent in millimols	0 07	0.08	0.35	0.41	0.35
NH$_3$-N produced, mgm.:					
In air	0.00	0.00	0 00	0.00	2.10
In medium	0 75	0 69	1 35	1.93	0.40
Total	0.75	0 69	1.35	1.93	2 50
Total, equivalent in millimols	0.05	0.05	0.10	0.14	0.18
Ratio CO$_2$/NH$_3$-N:					
Milligrams	4.2	5.2	11.3	9.3	6.1
Millimols	1.3	1.6	3.6	3.0	1.9
Production CO$_2$ per cell per hour (mgm. × 10^{-11})	42–99	66–185	52	36	1.3
Production NH$_3$-N per cell per hour (mgm. × 10^{-11})	10–24	13–36	4 5	4.1	0.2

the varying growth rates at this period, as pointed out in an earlier paragraph, but lay somewhere between 42 and 99 mgm. $\times 10^{-11}$ per cell per hour for CO_2 and between 10 and 24 mgm. $\times 10^{-11}$ for NH_3–N. This phase, which we have called a lag period, undoubtedly included some minutes of logarithmic increase toward its close. The presence of a stimulating salt, 0.1 M NaCl (column 2) caused a more rapid increase of bacteria (to nearly three fold the original number), and a slightly greater production of both CO_2 and NH_3-N per cell per hour, the increase being definitely more marked in the case of CO_2 than in that of NH_3–N We are not inclined to attribute great weight to these slight differences. On the whole, the results with and without stimulating salt check each other rather closely.

During the phase of logarithmic increase (2.2 to 4.0 hours) the bacterial population in the plain peptone medium (Column 3) multiplied more than twelvefold and the medium reverted to pH 7.0. Carbon dioxide and ammonia were both actively produced (the latter again only in the medium content). The calculated amount of CO_2 produced per cell was 52 mgm. $\times 10^{-11}$ per hour, lying within the range for the lag period. Ammonia production per cell per hour was 4.5 $\times 10^{-11}$ mgm., much less than the minimum for the lag period, and the ratio of CO_2 to NH_3–N on the basis of millimols rose to 3.6.

The logarithmic phase with stimulating salt (column 4) showed a much more rapid rate of increase (a thirty-fold multiplication in two hours). The carbon dioxide production per cell per hour was lower than in the plain peptone medium (36 $\times 10^{-11}$ mgm. as against 52) and the production of NH_3–N per cell per hour was about the same (4.1 $\times 10^{-11}$ mgm.).

Finally, between the twenty-third and twenty-seventh hours in plain peptone (column 5), the bacterial population remained nearly stable, increasing only by one-third. The pH by this time had risen to 8.3. Carbon dioxide was abundant in the air drawn through the apparatus but there was a decrease in the medium itself, registered in each one of the 4 experiments. In fact, the "production tension" of carbon dioxide excretion by the cells seems to have fallen below equilibrium with the rate of removal of the gas by aeration and therefore part of the observed aerated yield was actually due to the washing out from the medium of an accumulated excess built up in a previous period of more rapid production. Since the aerated yield contained this component which had not been actually produced during the four hours of its collection, this negative medium yield was subtracted from the observed aerated value.

TABLE 3

Average results in lactose-peptone medium

	PHASE OF		
	Lag	Logarithmic increase	Peak stability
	(1)	(2)	(3)
Period studied, hours after original inoculation	0–2 1	2–4	23.0–27.5
Initial bacterial population $\times 10^9$	1 21	3 09	100
Change in bacterial population (b/B)	2 6	10 5	0 9
Reaction, pH:			
Initial	6 7	6 4	4 8
Terminal	6 4	5 6	4 7
CO_2 produced, mgm.:			
In air	1 82	13 30	6 15
In medium	0.98	−0 09	0 14
Total	2 80	13.21	6 29
Total, equivalent in millimols	0.06	0 30	0 14
NH_3-N produced, mgm.:			
In air	0 00	0.00	0.01
In medium	0 44	0.10	0 07
Total	0.44	0.10	0 08
Total, equivalent in millimols	0.03	0 01	0.01
Ratio CO_2/NH_3-N:			
Milligrams	7.2	132	79
Millimols	2.3	42	25
Production CO_2 per cell per hour (mgm. $\times 10^{-11}$)	41–104	54	1.5
Production NH_3-N per cell per hour (mgm. $\times 10^{-11}$)	6–16	0 5	<0.1

Ammonia was abundant in both air and medium. The ratio of CO_2 to NH_3–N in millimols was about 2.0. The production of both CO_2 and NH_3–N per cell was reduced to a very low figure as compared with the earlier phases of the life cycle,—1.3×10^{-11} mgm. of CO_2 and 0.2×10^{-11} mgm. of NH_3–N per cell per hour.

Lactose-peptone medium. The data for three conditions in the lactose-peptone medium are presented in table 3.

Considering first the lag phase, we may compare column 1 of table 3 with column 1 of table 2. The rate of bacterial increase was very slightly higher in the presence of lactose. The reaction became slightly more acid. The most surprising finding is that while the production of ammonia was reduced in the presence of lactose (6 to 16×10^{-11} mgm. per cell per hour against 10 to 24) the production of carbon dioxide was the same (41 to 104×10^{-11} mgm. per cell per hour as against 42 to 99). The molecular ratio of CO_2/NH_3–N was 2.3 instead of 1.3 but this higher ratio was due solely to decreased ammonia formation.

In the phase of logarithmic increase (column 2 in table 3 and column 3 in table 2) the lactose-peptone medium became more acid (pH 5.6) while the peptone medium became more alkaline (pH 7.0). The bacterial increase, however, was about the same. Again, the rate of CO_2 production was the same in the two media (54×10^{-11} mgm. per cell per hour as against 52). The rate of ammonia production was very greatly decreased (0.5×10^{-11} mgm. per cell per hour as against 4.5) and the molecular ratio was consequently increased to 42 instead of less than 4.

It may be noted that our figures for ammonia production during this period were small and erratic; but they appear consistent with those for other periods. In the phase of peak stability (column 3 of table 3 and column 5 of table 2) the lactose-peptone had become so acid as pH 4.7, compared to an alkalinity of pH 8.4 in plain peptone. Despite the different reactions, the rates of CO_2 production per cell per hour were still very similar, being now at the low values of 1.5 and 1.3 mgm. $\times 10^{-11}$ respectively. On the other hand, ammonia production had fallen almost to the vanishing point in lactose-peptone, the total yield averaging less than 0.1 mgm. per 100 cc. as compared with 2.5 mgm. in peptone. Due to the very low and variable ammonia yields for this period in lactose-peptone, the rate of NH_3–N production and the ratios for CO_2/NH_3–N are here only of relative rather than absolute value.

Dolloff medium. Finally, table 4 shows the results obtained in the Dolloff synthetic medium, with and without a toxic salt concentration. In the plain Dolloff medium, our aerated cultures showed a period of initial stability prolonged for more than twenty-four hours, while in the presence of 1.5 M NaCl the population decreased at an approximately logarithmic rate (see fig. 1). The significance of these results from the standpoint of the

TABLE 4

Average results in Dolloff medium with and without toxic salt

	PHASE OF	
	Initial stability—Dolloff medium	Decrease—Dolloff medium plus 1.5 M NaCl
	(1)	(2)
Period studied, hours after original inoculation	0–25	0–24
Original bacterial population × 10^9	2.72	21.07
Change in bacterial population, b/B	0.8	0.1
Reaction, pH:		
Initial	6.2	5.9
Final	6.0	5.9
CO_2 produced, mgm.:		
In air	1.60	0.18
In medium	0.00	0.12
Total	1.60	0.30
Total, equivalent in millimols	0.04	0.01
NH_3-N produced, mgm.:		
In air	<0.01	0.00
In medium	<0.01	0.37
Total	<0.01	0.37
Total, equivalent in millimols		0.03
Ratio CO_2/NH_3-N:		
Milligrams		0.8
Millimols		0.3
Production CO_2 per cell per hour, mgm. × 10^{-11}	2.9	0.2
Production NH_3-N per cell per hour, mgm. × 10^{-11}	<0.1	0.3

bacterial growth cycle has been discussed elsewhere (Winslow, Walker and Sutermeister (1932)).

The data presented in column 1 of table 4 show that the long period of stable population in the Dolloff medium is not to be considered as a prolonged lag period but as a resting phase analogous to the period of peak stability which succeeds the phase of logarithmic increase in other media. It will be noted that during a period of twenty-five hours the bacterial population decreased only by 20 per cent. The reaction (which was initially rather acid in this synthetic medium) changed only from pH 6.2 to pH 6.0. The production of CO_2 was 2.9×10^{-11} mgm. per cell per hour, comparing with 1.5 for lactose-peptone and 1.3 for plain peptone during the period of peak stability. The ammonia production was less than 0.1×10^{-11} mgm. per cell per hour as in

TABLE 5

Comparative results in various media and at various phases of the population cycle

	MEDIUM	PHASE OF			
		Lag	Logarithmic increase	Peak stability	Logarithmic decrease
CO_2 production, (mgm. $\times 10^{-11}$ per cell per hour)	Peptone	42–99	52	1.3	
	Peptone plus 0.1 M NaCl	68–185	36		
	Lactose-peptone	41–104	54	1.5	
	Dolloff			2.9*	
	Dolloff plus 1.5 M NaCl				0.2
NH_3-N production, (mgm. $\times 10^{-11}$ per cell per hour)	Peptone	10–24	4.5	0.2	
	Peptone plus 0.1 M NaCl	13–36	4.1		
	Lactose-peptone	6–16	0.5	<0.1	
	Dolloff			<0.1*	
	Dolloff plus 1.5 M NaCl				0.3
Ratio CO_2/NH_3-N (millimols)	Peptone	1.3	3.6	1.9	
	Peptone plus 0.1 M NaCl	1.6	3.0		
	Lactose-peptone	2.3	42	25	
	Dolloff				
	Dolloff plus 1.5 M NaCl				0.3

* This phase, which is chronologically lag but functionally much like peak stability, has been termed in the text "initial stability."

the lactose-peptone medium at peak stability and as compared with 0.2 mgm. in the plain peptone at a corresponding phase. The actual amount of ammonia formed in this case was too small to permit a valid computation of CO_2/NH_3–N ratios.

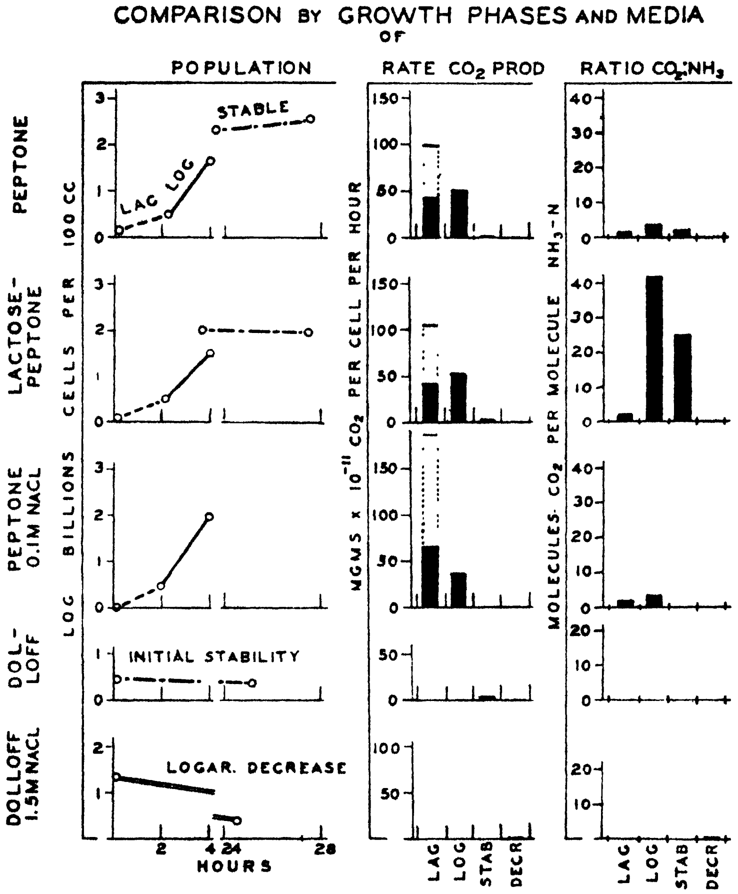

Fig. 2. Comparative Population Changes, Rates of CO_2 Production per Cell per Hour and Molar Production Ratios of CO_2/NH_3-nitrogen by Growth Cycle Phases in Various Media

In Dolloff solution to which a toxic salt concentration (1.5 M NaCl) had been added and in which a very heavy initial inoculum was used, the bacteria died off at such a rate that only one-tenth of the original number were left after twenty-four hours. The reaction remained at pH 5.9. The CO_2 production reached the very low figure of 0.2×10^{-11} mgm. per cell per hour while the NH_3–N equalled 0.3×10^{-11} mgm. This is the only instance in which ammonia production exceeded carbon dioxide production. The actual amounts of carbon dioxide were, however, too small for accurate determination and, while the amounts of ammonia were actually large, they were small in relation to the high ammonia content of the medium. We do not believe, therefore, that these data can be stressed. So far as they may be considered significant, they suggest that we are dealing in this case with autolysis of dead cells rather than metabolism of living ones.

Comparative results in different media. The principal comparative results in different media and at different phases of the life cycle are summarized for convenience in table 5. The rates of bacterial increase, the rates of CO_2 production per cell per hour and the molar ratios of carbon dioxide to ammonia are presented graphically in figure 2.

SUMMARY OF CONCLUSIONS

1. During a period of stable population in a continuously aerated medium of plain 1 per cent peptone water, *Esch. coli* produced 1.3×10^{-11} mgm. of CO_2 per cell per hour and 0.2×10^{-11} mgm. of NH_3–N. This corresponds to a splitting off from the peptone of approximately two molecules of CO_2 for each molecule of ammonia nitrogen. This period may perhaps be considered as representing something analogous to a condition of basal metabolic activity of the bacterial cell.

2. During a similar period of peak stability in a medium containing 0.5 per cent peptone and 0.5 per cent lactose, the rate of production of NH_3–N was substantially decreased (to far less than 0.1×10^{-11} mgm.) while the rate of production of CO_2 remained substantially unchanged (1.5×10^{-11} mgm.). In a synthetic medium containing lactose and ammonium tartrate as nutrients the rate of production of CO_2 was only a little greater (2.9 mgm. $\times 10^{-11}$ per cell per hour) while the rate of NH_3–N production was again sharply reduced.

These results quantitatively confirm the conclusions of Kendall and others in regard to the nitrogen-sparing effect of carbohydrates. The molecular ratio of CO_2 to NH_3–N rises in lactose-peptone water to 10 to 20 times the ratio in plain peptone water. The surprising thing, however, is that the sparing of protein is accomplished with no important increase in CO_2 formation. The increased ratio of CO_2 to NH_3-N is due to lessened formation of NH_3–N rather than to greater production of CO_2. The actual liberation of CO_2 per cell per hour from peptone alone is about the same as when half the peptone is replaced by lactose.

Furthermore, the rate of bacterial increase and the total CO_2 production even early in the culture activity are approximately the same whether lactose is present or not, as indicated below. On the other hand, reduction of NH_3 yield when lactose is added appears from the start.

MEDIUM	HOURS AFTER ORIGINAL INOCULATION	INITIAL BACTERIAL NUMBERS $\times 10^9$	CHANGE IN BACTERIAL NUMBERS, b/B	TOTAL CO_2 FORMED	TOTAL NH_3N FORMED
				mgm.	*mgm.*
Peptone	0–2 2	1 45	2.3	3.15	0.75
Lactose-peptone	0–2 1	1.21	2 6	2 80	0 44
Peptone	2.2–4	3.38	12.7	15 23	1 35
Lactose-peptone	2–4	3 09	10 5	13 21	0 10

This suggests that the phenomena observed in the ordinary fermentation tube (visible accumulation of gas in closed arm in a lactose medium but not in a peptone medium) are due to differences in reaction, in solubility and in diffusion of CO_2 into the atmosphere rather than to differences in the actual production of CO_2.

3. Toward the end of the initial lag period in either peptone or lactose-peptone water, there is manifest an enormous increase in metabolic activity, particularly with respect to ammonia production. Formation of CO_2 per cell per hour is increased thirty to seventy fold and formation of NH_3–N fifty to one hundred and fifty fold as compared with the peak stability rates (paragraphs 1 and 2). There is in these rates a very clear demonstration of

the physiological youth of the bacterial cells as postulated by Sherman and Albus (1923). The molecular ratio in this period suggests that in plain peptone one molecule of CO_2 is formed for each molecule of NH_3–N.

4. During the phase of logarithmic increase, the average rate of metabolic activity is somewhat slower than at the close of the lag phase but still much more rapid than in a phase of stable population. The actual rate in the peptone medium is 52 mgm. $\times 10^{-11}$ of CO_2 per cell per hour (40 times the figure for a stable population) and 4.5×10^{-11} mgm. of NH_3–N (20 times the figure for a stable population). The molecular ratio is 4. In the lactose-peptone medium, the rate of CO_2 production per cell per hour is 54×10^{-11} mgm. (36 times the figure for a stable population), but the rate of NH_3–N formation is only 0.5 and the yields give the enormous molecular ratio of 42. It is apparent that when active formation of new cells is going on, the liberation of CO_2 is relatively very rapid as might be expected from the fact that nitrogenous products of decomposition are at this time being utilized in the building of new protoplasm.

5. The addition of a stimulating salt (0.1 M NaCl) to the peptone medium does not markedly affect ammonia-production but appears to increase the rate of CO_2 formation during the lag phase and to decrease it during the phase of logarithmic increase. This phenomenon may be in part affected by the difficulty of drawing an exact boundary between the lag and logarithmic phases.

6. The addition of a toxic salt (1.5 M NaCl) to a simple synthetic medium gives yields of CO_2 and NH_3–N too small for accurate determination; but an apparent excess of NH_3–N over CO_2 suggests that autolysis rather than metabolism is the dominant feature of the process in this case.

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FACTORS DETERMINING THE RATE OF MORTALITY OF BACTERIA EXPOSED TO ALKALINITY AND HEAT[1]

J. H. WATKINS AND C.-E. A. WINSLOW

Department of Public Health, Yale School of Medicine

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OBJECTIVES OF STUDY

Since the basic study of Krönig and Paul (1897), it has been recognized that the mortality of bacteria exposed to unfavorable environmental conditions follows a generally regular and consistent course. Under a wide variety of conditions, this course is such that, when the number of bacteria surviving at a given moment is plotted against the logarithm of the elapsed time since the beginning of the experiment, we obtain a straight line. In a later paragraph we shall return to the conflict of opinion as to the exactness with which this relation holds and as to its theoretical significance. The general phenomenon is, however, recognized by all students of the subject.

There has been, so far as we are aware, but one really outstanding exception to the general rule. Levine, Buchanan and Lease (1927) and Levine, Buchanan and Toulouse (1927), working with an unidentified spore-former exposed to alkalies, found that the velocity coefficients of disinfection were not uniform throughout the course of an experiment but progressively increased with the time of exposure. Myers (1929) repeated this work, using the same organism and obtained confirmatory results.

The present study was originally undertaken with a view to determining whether the same phenomenon could be observed when alkaline solutions acted upon vegetative cells. It seemed possible that in the action of alkali upon spores there might be two different factors involved,—one related to penetration through the resistant spore-wall, and one to direct toxic effects upon protoplasm. If this were the case, the action of alkalies upon vegetative cells might fail to show the anomalous behavior reported by Levine for spores.

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[1] Based on a thesis presented by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Yale University.

In the course of our studies the observation of discordant results when parallel experiments were compared led us to a search for the cause of such discrepancies. We have found at least two factors which appear of prime importance,—the initial concentration of bacteria used and the age of the stock culture from which these bacteria are obtained.

TECHNIQUE

Organism. The organism used was a stock culture of *Esch. coli* (communis type) which has been extensively used in this laboratory for viability experiments during the past ten years (for instance by Cohen (1922); Falk and Winslow (1926); Winslow and Brooke (1927); Fabian and Winslow (1929)). It was maintained on agar slants and transferred daily at room temperature. For the actual experimental work, our inoculum was obtained from twelve to eighteen-hour growth on agar in a Kolle flask incubated at 37°C. The material for the inoculum was washed off in sterile distilled water, washed three times by centrifugation at high speed in sterile distilled water and filtered through cotton. A series of special tests showed that cells washed by six centrifugations gave mortality rates identical with those obtained after three washings. Therefore, it was concluded that the triple centrifugation would remove protective substances carried over from the agar culture so far as it was practical to do so.

Toxic solutions. The bacteria were exposed to the toxic solutions in a 500 cc., three-hole, round-bottomed Woulff flask, brought to a constant temperature and maintained at that temperature with constant stirring in a water-bath.

The chemical disinfectant employed was NaOH, made up to desired concentration from a saturated solution which had stood in a high cylinder for several weeks to clear itself of suspended carbonate. In our first experiments, the test solutions were made immediately before use while the cultures were being washed. It was hoped by this method to reduce to a minimum possible changes in the hydroxyl ion concentration through the absorption of atmospheric CO_2. Subsequent experiments, however, showed that this effect was negligible with the solutions and time periods in use and the disinfecting solutions were made up thereafter by dilution from a previously prepared and standardized N/10 solution which was kept in a Pyrex flask in the ice box and used through one series of experiments. Standardization was carried out by means of N/10 sulfuric acid prepared against a weighed concentration of potassium acid phthalate according to the method of Clark (1928).

In most of our experiments we used a solution containing only NaOH in distilled water. In one series, we studied the effect of a buffer mixture suitable for killing within the range of desired temperature and time, a Sørensen borate-NaOH mixture, prepared after Walbum as described by Clark (1928). Five parts of borate solution (12.404 grams boric acid dissolved in 100 cc. of CO_2-free normal NaOH and diluted to 1 liter) were added to five parts of exact tenth normal CO_2-free NaOH. The pH of this solution as given by Clark was 10.82; after standing overnight the pH of our solution as determined colorimetrically by means of LaMotte-purple indicator was 10.8. This solution was made in sufficient volume at one time to last throughout the course of the experiments.

The purpose of using this buffer solution was to obtain the disinfecting action of the OH ion without possible (though improbable), interference by CO_2 absorbed from the atmosphere.

It was necessary, of course, in the experiments with NaOH and the buffer solution, to stop the killing action in the proportion withdrawn for plating by the use of suitable neutralizing solutions. For this purpose 50 cc. of a sterile H_2SO_4 solution, of normality one-tenth as strong as the disinfectant, were used for the NaOH experiments. For example, when N/200 NaOH was used, 5 cc. were withdrawn at stated intervals into 50 cc. of sterile N/2000 H_2SO_4, prepared by dilution of a tenth-normal solution. An indicator (methyl orange) was added to determine whether approximate neutrality had been reached when the solutions were mixed. In the case of the buffer solutions, it was found experimentally that 50 cc. of the following solution would bring 5 cc. of the buffer solution to the color indicating pH 7.1:

	cc.
Peptone, 1 per cent	1,000
Citric acid (0.1 molar)	32
Brom thymol blue (0.4 per cent)	5

The neutralized solution supported a strong growth of *Esch. coli.* when inoculated and incubated overnight at 37°C. which would indicate that no harm was done the cells by the neutralizing solution during the short time they remained in it.

Heat treatment. In certain experiments we used heat alone as the killing agent. In these cases the inoculum was suspended in sterile distilled water which was obtained by double distillation, first in a Barnstead still and second through a Liebig condenser. Since the killing agent in this case was heat, inoculation of the 5 cc. portions into cold 99 cc. sterile water blanks accomplished sufficiently well the purpose of interrupting the killing process.

Water-bath. As stated above, a constant temperature water-bath was used throughout the experiments. The water, in an insulated chamber, was heated by large carbon lamps controlled by means of a battery relay and mercury thermostat, and was kept in constant circulation by means of a current of air supplied at the bottom of the bath and near the heating units. The range of sensitivity was 0.05°C. as determined by a United States Bureau of Standards thermometer. The killing agent, in the Woulff flask, was kept in a constant state of movement by a sterile glass stirrer inserted in the central hole through a rubber stopper and turned by an electric motor slowly enough to keep bubbles from forming at the sides of the flask. A thermometer, sterilized by means of a flame and alcohol, was inserted in another hole. The third hole was used for the withdrawal of the sample. As some trouble was encountered in keeping the pipette from touching the side of the flask neck, a short length of glass tubing (of sufficient size to admit the stem of the pipette) was inserted in the rubber stopper, with its end plugged with cotton to insure against inadvertent transfer to the outside of the pipette of organisms not subjected to complete disinfecting action. A number of these special stoppers were prepared and a new one inserted in the neck of the Woulff flask immediately after each sample had been withdrawn.

Plating and incubation. When the time interval permitted, the neutralized sample was plated at once into sterile Petri dishes. Duplicate plates were made over a range of several dilutions, and triplicate plates for the dilutions most likely to be significant. The plates were poured with nutrient agar and as soon as solidification had occurred, were incubated for forty-eight hours at 37°C. In case the time intervals between withdrawals were insufficient for immediate plating, the plating was done as soon as the experiment was completed. The organisms seldom remained longer than fifteen minutes in the neutralized solution before plating.

Counting was done in a Buck colony counter containing a Jeffer counting plate.

Time of exposure. The period of exposure with the NaOH solutions and with water at 55° was generally from twenty to eighty minutes (in 10 instances, less than twenty minutes with a minimum of six minutes). With borate-buffer mixture it was nine to forty-two minutes; and with water at 50°, one hundred to one hundred twenty minutes.

Extent of study. Altogether, 151 different experiments were made, distributed as follows:

	Number of experiments
N/200, NaOH, 30°C	26
N/150, NaOH, 30°C	16
N/100, NaOH, 30°C	32
N/125, NaOH, 22°C	23
N/100, NaOH, 22°C	12
Borate buffer mixture, 30°C	16
Distilled water, 55°C	15
Distilled water, 50°C	11

GENERAL FORM OF MORTALITY CURVE

It has been noted that most students of disinfection have recognized the generally logarithmic course of the reduction in living cells (Madsen and Nyman (1907); Chick (1908); Chick and Martin (1908); Hewlett (1909); Chick (1910); Chick (1912); Paul (1909); Paul, Birstein and Reuss (1910); Phelps (1911); Reichenbach (1911 and 1922); Robertson (1914); Hinds (1915); Lee and Gilbert (1918); Cohen (1922); Gates (1929); Bancroft and Richter (1931)). Most of the workers cited have assumed that the regularity of this reduction was due to fundamental chemical or physical factors which involved some basic process analogous to a monomolecular reaction. Some, while accepting the essentially logarithmic form of the curve held that the monomolecular analogy was descriptive only and not explanatory.

Certain of these investigators, and others, have pointed out that the ideal curve of logarithmic decrease is by no means always completely realized. In certain cases, there may be marked deviations from the logarithmic curve, particularly at the beginning and end of the process (Chick (1908 and 1912); Eijkman (1908, 1909 and 1912); Reichenbach (1911 and 1922); Brooks (1918); Smith (1921); Gates (1929); Knaysi (1930a, 1930b and 1930c); Knaysi and Gordon (1930)). In general, these workers observed curves of a sigmoid shape, involving a diminished rate of reduction toward the end of exposure and tended to explain the observed phenomena by biological variability in the exposed cells. Fulmer and Buchanan (1923) held any resemblance to a monomolecular reaction to be "superficial and fortuitous." Falk and Winslow (1926) and Rahn (1929 and 1931) explained the observed deviations from a logarithmic rate of reduction on physico-chemical grounds without invoking the assumption of biological variation.

In general, all the investigators cited, whatever their theoretical explanations, admitted that the logarithmic rate of decrease normally prevails for a major part of the period of exposure to unfavorable conditions. Levine, Buchanan and Lease (1927), Levine, Buchanan and Toulouse (1927) and Myers (1929), however, concluded that spores exposed to alkali showed a wholly different phenomenon,—the velocity coefficients of the death rate increasing progressively with the time of exposure.

TABLE 1

Typical set of experiments (N/200 NaOH, 30°C.)

TIME	BACTERIA PER CUBIC CENTIMETER	LOGARITHMS	LOGARITHMS, PER CENT SURVIVING	K
Experiment 6				
0	55,400,000	7.743	2.000	
10	41,300,000	7.616	1.872	0.013
20	36,100,000	7.558	1.814	0.009
30	27,200,000	7.435	1.692	0.010
40	24,400,000	7.387	1.644	0.009
50	15,400,000	7.188	1.444	0.011
60	9,650,000	6.985	1.241	0.013
70	9,650,000	6.985	1.241	0.011
80	6,680,000	6.825	1.083	0.011
				0.011
Experiment 7				
0	2,550,000	6.407	2.000	
10	1,030,000	6.013	1.608	0.039
20	388,000	5.589	1.185	0.041
30	113,000	5.053	0.647	0.045
40	38,400	4.584	0.179	0.046
50	3,710	3.569	−1.161	0.057
60	1,640	3.215	−2.808	0.053
				0.047
Experiment 8				
0	350,000	5.544	2.000	
5	303,000	5.481	1.937	0.013
10	179,000	5.253	1.709	0.029
15	74,000	4.869	1.324	0.045
20	39,400	4.596	1.053	0.047
30	8,600	3.935	0.391	0.054
40	975	2.989	−1.446	0.064
				0.042

Our primary objective was to see whether the same condition would obtain when vegetative cells were exposed to alkali. As noted above, we have 109 different experiments with pure NaOH, 16 with borate buffer mixtures and 26 with heat alone as controls. The basic data for three of these experiments (taken quite at random from our notes) are presented in table 1.

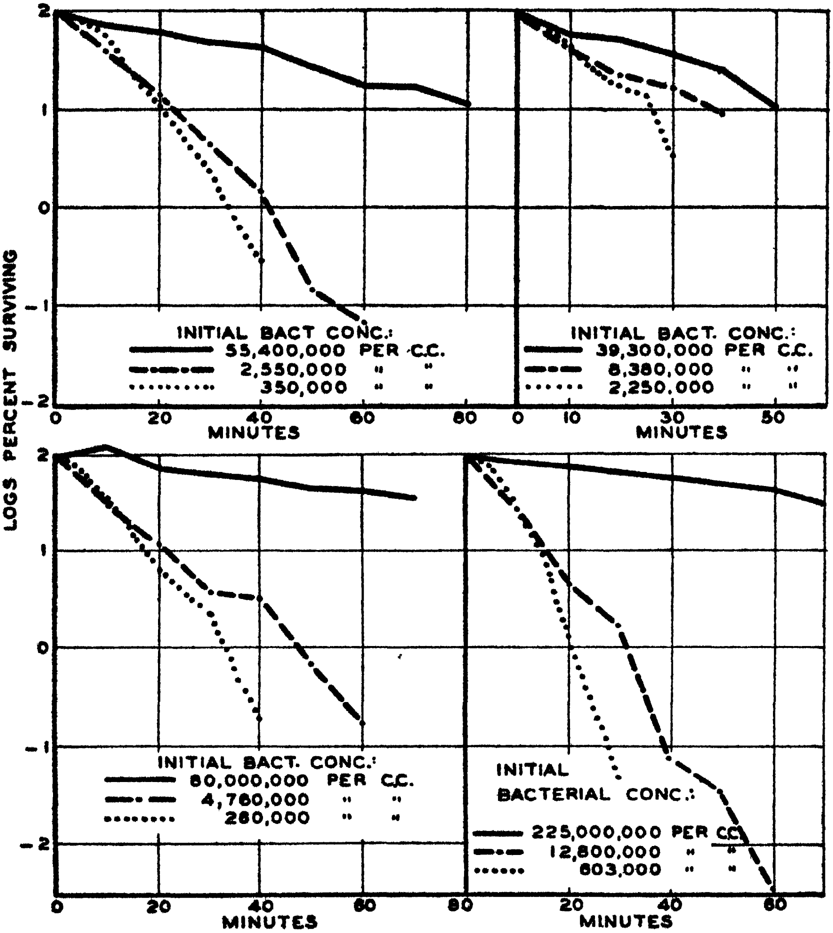

FIG. 1. SURVIVORSHIP CURVES OF *Esch. coli* IN N/200 NaOH AT 30°C.

Curves have been plotted for all the 151 experiments, and a typical group of such curves is presented in figure 1.

We analyzed our 151 curves in two ways. First, by inspection, each curve was classed as either essentially linear, parabolic and convex upward, parabolic and convex downward or sigmoid. The results are presented in the first columns of table 2. They show that 62 per cent of all the NaOH curves, 81 per cent of the buffer-mixture curves and 58 per cent of the hot-water curves were of linear form, while 25 per cent of the NaOH curves and 35 per cent of the hot-water curves were parabolas convex upward. The latter form would indicate an increasing rate of mortality with prolonged exposure and would conform to the phenomenon reported by Levine. Only in the 2 cases of N/200 NaOH and 55° hot water did this type of curve appear to predominate. The deviations from a linear curve were in all cases slight and our classification by inspection was largely a matter of subjective judgment. For instance, of the curves presented in figure 1, six were classed as linear, and six as convex upward. It will be noted that the deviations are all exceedingly slight and may well be due to chance.

TABLE 2

General summary of logarithmic survivorship curves. All experiments

DISINFECTANT AGENT	FORM OF CURVE				TOTAL	RATIO OF K FOR FIRST HALF OF PERIOD TO K FOR SECOND HALF
	Linear	Parabolic		Sigmoid		
		Convex upward	Convex downward			
N/200 NaOH, 30°	8	18	0	0	26	0.93
N/150 NaOH, 30°	14	1	0	1	16	1.03
N/100 NaOH, 30°	24	4	0	4	32	0.88
N/125 NaOH, 22°	19	1	1	2	23	1.12
N/100 NaOH, 22°	3	3	3	3	12	0 94
Total NaOH	68	27	4	10	109	0 97
Borate-buffer mixture	13	0	3	0	16	1.17
Water, 50°	11	0	0	0	11	1.23
Water, 55°	4	9	0	2	15	0 77
Total water	15	9	0	2	26	0.96

A more accurate evaluation of the process was then obtained as follows. First, the actual rate of decrease for each period was computed from the formula:

$$K = \frac{\log b - \log B}{t}$$

where b = number of bacteria at beginning of time interval.
B = number of bacteria at end of interval.
t = length of interval.

These results are given, for example, in the last column of table 1.

We then averaged the values of K for the first and second halves of each experiment and divided the average for the first half by that for the second. A ratio of 1 would indicate a constant logarithmic rate of reduction and a ratio below 1 would indicate the increasing rate of reduction reported by Levine.

It will be noted from the last column in table 2 that the ratios closely approximate unity and approach unity more closely the larger the group concerned. Furthermore, there is no difference between the results of disinfection with NaOH and with hot water. It would appear then that the killing of vegetative cells by alkali does not follow the course observed by Levine in the case of spores.

EFFECT OF INITIAL CELL CONCENTRATION UPON RATE OF MORTALITY

Early in the course of these experiments it was noted that the rate of reduction in bacterial numbers was very definitely related to the concentration of the original inoculum. This fact is clearly illustrated for example in figure 1. The general phenomenon of increased survival when a heavy inoculum is used is of course by no means a new one, having been noted or implied by Geppert (1889, a and b), Behring (1890), Boer (1890), Abbott (1891), Frankland (1895), Ficker (1898), Bellei (1904), Jordan, Russell and Zeit (1904), Houston (1908), McClintic (1912), Wright (1917), Bigelow and Esty (1920), Smith (1921), Weiss (1921), Klieneberger (1924), and Lockemann and Picker (1928). In many instances the investigators cited were merely observing the effect of initial bacterial numbers upon ultimate survival and the phenomena were explicable as due to the chance inclusion of more resistant forms in a larger original group. In at least three instances, however, (Paul, Birstein and Reuss, 1910, Salter, 1921 and Ballantyne, 1930) the data presented make it possible to compute rates of decrease for definite experiments and to show that in each observer's work, the coefficient of decrease actually varied with initial numbers.

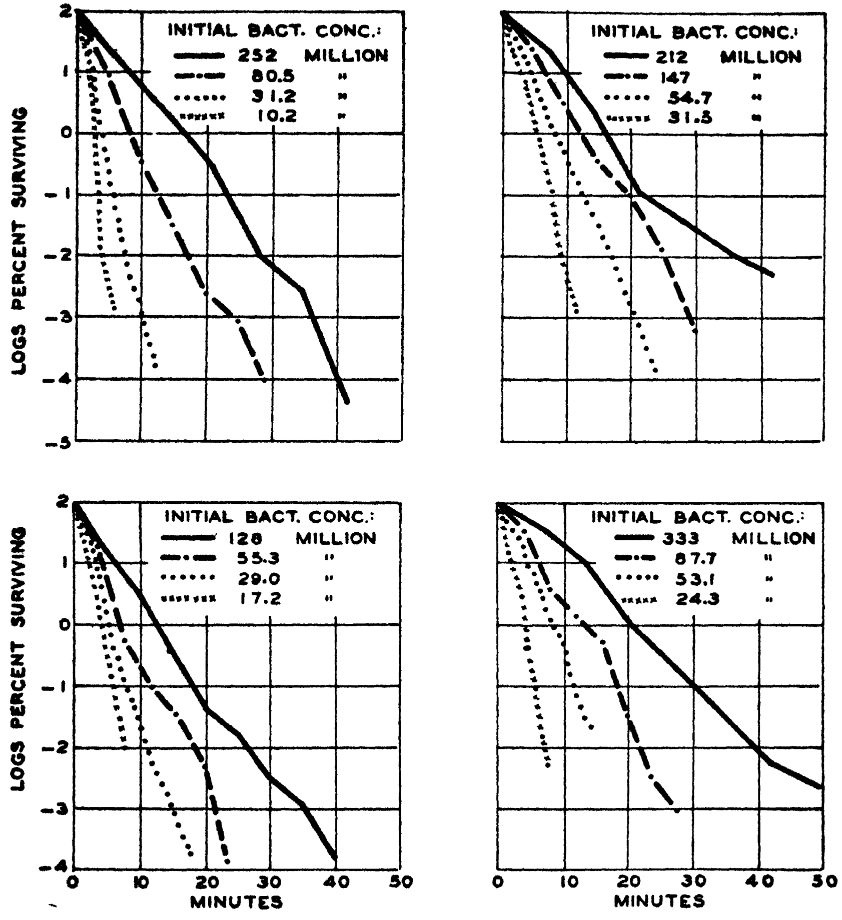

FIG. 2. SURVIVORSHIP CURVES OF *Esch. coli* IN N/100 NaOH AT 30°C.
(Culture age, fourteen hours)

The trend of our own results has been already suggested in figure 1 and may be further illustrated in figure 2 which presents the data for four specially significant series of experiments in which cultures of uniform age (fourteen hours) were used for the inoculum and N/100 NaOH at 30° as the disinfectant agent. In each series of experiments, four different concentrations of bacteria were used for the initial inoculum and it will be noted how definitely the slope of the survivorship curve tends to become more abrupt with smaller and smaller initial numbers.

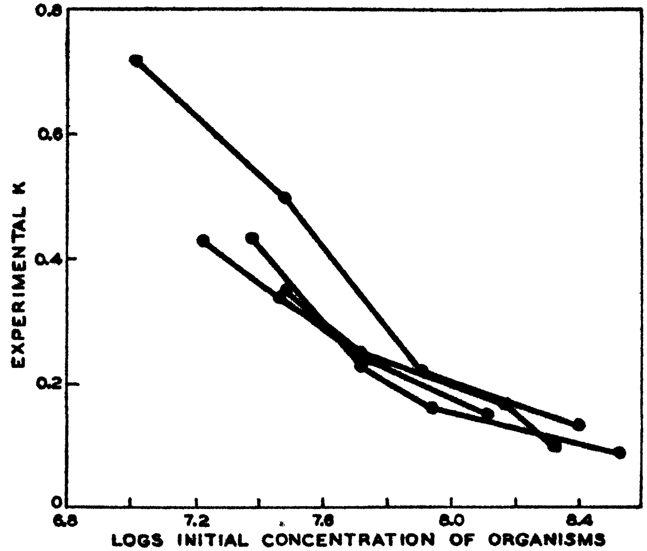

FIG. 3. RELATION BETWEEN INITIAL CONCENTRATION OF ORGANISMS AND AVERAGE EXPERIMENTAL K

N/100 NaOH, 30°C.; fourteen-hour cultures

Figure 3 shows how, when these four series of experiments are combined the coefficient K decreases with increasing concentration.

In table 3 we have tabulated the average figures for K in all our experiments classified in groups according to the initial concentration of organisms present. The two sets of experiments in which heat was the disinfecting agent were included to see whether the protective effect of numbers was exerted with respect to a physical as well as a chemical agency.

It will be noted by reference to table 3:

That at a given temperature (30° or 22°) the value of *K* increases as the concentration of NaOH increases. Doubling the concentration increases *K* tenfold.

That with a given concentration of NaOH (N/100) the value of *K* increases with higher temperature. An 8° rise in temperature increases *K* by from 35 to 109 per cent, the maximum effect appearing with low cell concentrations.

That the borate-buffer mixture is about as toxic as N/100 NaOH, somewhat less so with low cell concentrations, somewhat more so with high cell concentrations.

That hot water at 50° is only slightly toxic for the organism studied but that hot water at 55° is about as toxic as N/150 NaOH at 30°C.

That increasing the temperature of hot water by five degrees (from 50° to 55°) increases *K* twenty to thirtyfold.

TABLE 3

Rate of Mortality (K) in Relation to Initial Concentration of Organisms (Average K for all experiments having an inoculum in specified range)

	TEMPERATURE	INITIAL CELL CONCENTRATION (MILLIONS BACTERIA PER CUBIC CENTIMETER)			
		Over 100	50–99	1–49	Under 1
N/200 NaOH	30°	0.015	0.018	0.045	0.061
N/150 NaOH	30°	0.055	0.112	0.150	
N/100 NaOH	30°*	0.128	0.217	0.465	
N/125 NaOH	22°	0.051	0.062	0.120	
N/100 NaOH	22°	0.095	0.122	0.222	
Borate-buffer	30°	0.280	0.323	0.402	
Water	50°	0.003		0.006	
Water	55°	0.088	0.082	0.118	

* This line of averages is based not on the total of 32 experiments made with N/100 NaOH at 30° (see table 2) but on the 20 experiments made in this menstruum with fourteen-hour cultures and different initial cell concentrations. The other 12 experiments with N/100 NaOH at 30° were made with the same initial cell concentrations and cultures of different ages.

That in all instances (with one single exception in the case of water at 55°) a reduction in the number of bacterial cells in the original inoculum increases the value of K. Experiments starting with inocula of between one and fifty million cells per cubic-centimeter show values of K from two to three times higher than those starting with inocula of over 100 million cells per cubic centimeter under otherwise comparable conditions.

The last of these conclusions is the one in which we are particularly interested at the moment. It is evident that the adage, "there is safety in numbers" applies with surprising exactitude to bacterial populations.

When the disinfectant agent is chemical in nature, it is easy to conceive how this comes to pass. We need only assume that the bacterial cell eliminates into the surrounding menstruum products which tend to neutralize or inhibit the action of the disinfecting agent; for, if this were the case, the concentration of such protective substances would be increased in proportion to the nearness to each other of the cells producing them. Shaughnessy and Winslow (1927) have demonstrated that such is actually the case and that in an alkaline solution, for example, the bacterial cell liberates acidic substances which produce a measurable change in the surrounding menstruum as a whole.

The general effect of protective substances in a bacterial suspension has been discussed by Ficker (1898), Buchanan and Fulmer (1928), Winslow and Brooke (1927), Winslow (1928), Klarmann, Shternor and von Wowern (1929) and Ballantyne (1930). In nearly all instances, however, the protective substances have been artificially added to the menstruum. It seems to be clear from our washing experiments that such substances were not in our case carried over from the agar culture but must have been formed by the cells themselves in the disinfectant medium. Disinfection with both sodium hydroxide and heat was performed with suspensions of organisms which had been washed three times, and it was shown that three further washings had no marked effect upon either the slope or shape of the survivorship curves. The presence of materials in solution carried over from the original cultures which would effect rates of death must, then, be eliminated from consideration. If such substances are responsible for the protection of cells against death, their elaboration must take place as the cells are exposed to the killing action; or they must have been carried over with the cells in such manner that washing does not remove them.

It seems probable that dead as well as living bacterial cells may exert this regulatory influence upon their environment. Lange (1922) reported a protective effect due to dead cells. If dead cells were not active in this way one would expect that in such experiments as our own the value of *K* would progressively rise during the course of each experiment as concentration of living cells decreases; but, as we have seen, such is not generally the case.

It is easy, then, to understand how protective substances would safeguard bacteria against chemical disinfectants by such a direct neutralizing effect as was observed by Shaughnessy and Winslow (1927). That high initial concentration of cells should increase resistance to heat is much more surprising. Yet such an increase has been demonstrated by Lange (1922), Behrens (1923) and Hückel (1926), as well as in our own work.

This phenomenon cannot be explained by a direct physical effect of the bacterial cells upon the specific heat of the suspension under the actual conditions of such experiments as to temperature and time. We can only suggest the possibility that a zone of protective substances in the menstruum surrounding a given cell may react upon the chemical or physical condition of the cell wall itself in such a way as to make it more resistant to the influence of heat.

EFFECT OF AGE OF CULTURE USED AS SOURCE OF ORIGINAL INOCULUM

A preliminary analysis of our data indicated that even when allowance was made for the influence of the amount of the original inoculum, the values for the velocity coefficient *K* were not always consistent. As the next factor which might conduce to variability, we turned to the question of the age of the original inoculum.

This factor was referred to in more or less general terms by Esmarch (1889), Fraenkel (1889) and Behring (1890). Esty (1920), Esty and Meyer (1922), Magoon (1926) and Sommer (1930) have discussed the effect of age upon the resistance of spores. In respect to vegetative cells, Boer (1890), Reichenbach (1911 and 1922), and Sherman and Albus (1923) have made particularly important contributions. All of these investigators reported that the cells from very young cultures were less resistant than those from older ones. On the other hand, Ficker (1898), Chick (1908 and 1912) and Stark and Stark (1929, a and b) obtained opposite or variable results, depending on the ages and particular lethal conditions employed. In the case of Chick's experiments, inocula were made from very old cultures and there need be no necessary conflict between the findings of various observers. It may well be that a very young culture and a very old culture may both yield cells of low resistance as compared with a culture in active growth. The phenomenon may be analogous to the well-known fact that inoculation from a culture in the stage of logarithmic increase into a new tube of the same medium shows no lag phase (see Winslow (1928)).

In our own routine work, the Kolle flasks used for the inoculum were incubated over night and the age of these source-cultures, at the time exposure to lethal conditions was begun, varied from eight to nineteen hours,—although usually between fourteen and sixteen hours.

To test the effect of culture age we conducted a special series of 16 experiments. In all of them, the original inoculum was between 123 million and 230 million per cubic centimeters and the toxic agent was N/100 NaOH at 30°. The age of the original source culture only was varied, being eight, eleven, fourteen and seventeen hours, respectively.

The results of these experiments are presented in table 4 and in figure 4.

It is evident from inspection of the table and chart that even differences in culture age of as little as three hours exerts a clear and definite influence upon cell resistance. In figure 5, the average values of *K* are plotted against the age of the source-culture and the closeness of the relation exhibited is strikingly apparent.

Finally, to show the combined influence of the two variables studied (age of source-culture and amount of inoculum) we have plotted in figure 6 the results of 30 different experiments made with N/100 NaOH at 30° in which the exact age, as well as the amount of the original inoculum were known. This series, of course, includes the 16 experiments summarized in table 4 as well as the earlier experiments made with the same menstruum. In figure 6, the ordinates represent source-culture age and the abscissae, concentration of inoculum. For each experiment, a symbol has been set down on the chart at the appropriate point, the symbol indicating the value of *K* obtained for that particular

TABLE 4

Summary of effect of age of cultures upon average K (N/100 NaOH, 30°C.)

EXPERIMENT NUMBER	AGE OF CULTURE	BACTERIA PER CUBIC CENTIMETER	LOGARITHMS	AVERAGE *K*
	hours			
114	17	230,000,000	8.362	0.124
121	17	123,000,000	8.090	0.119
122	17	182,000,000	8.260	0.166
129	17	168,000,000	8.225	0.138
				0.137
115	14	198,000,000	8.297	0.136
120	14	173,000,000	8.238	0.139
123	14	215,000,000	8.332	0.191
128	14	185,000,000	8.267	0.148
				0.154
116	11	210,000,000	8.322	0.199
119	11	148,000,000	8.170	0.218
124	11	150,000,000	8.176	0.242
127	11	130,000,000	8.114	0.220
				0.220
117	8	132,000,000	8.121	0.336
118	8	127,000,000	8.104	0.478
125	8	185,000,000	8.267	0.344
126	8	157,000,000	8.196	0.362
				0.380

experiment. It will be noted that the lowest values of *K* (indicated by vertical-horizontal crosses) all lie in the upper right corner of the chart and that as one passes to the left or downward

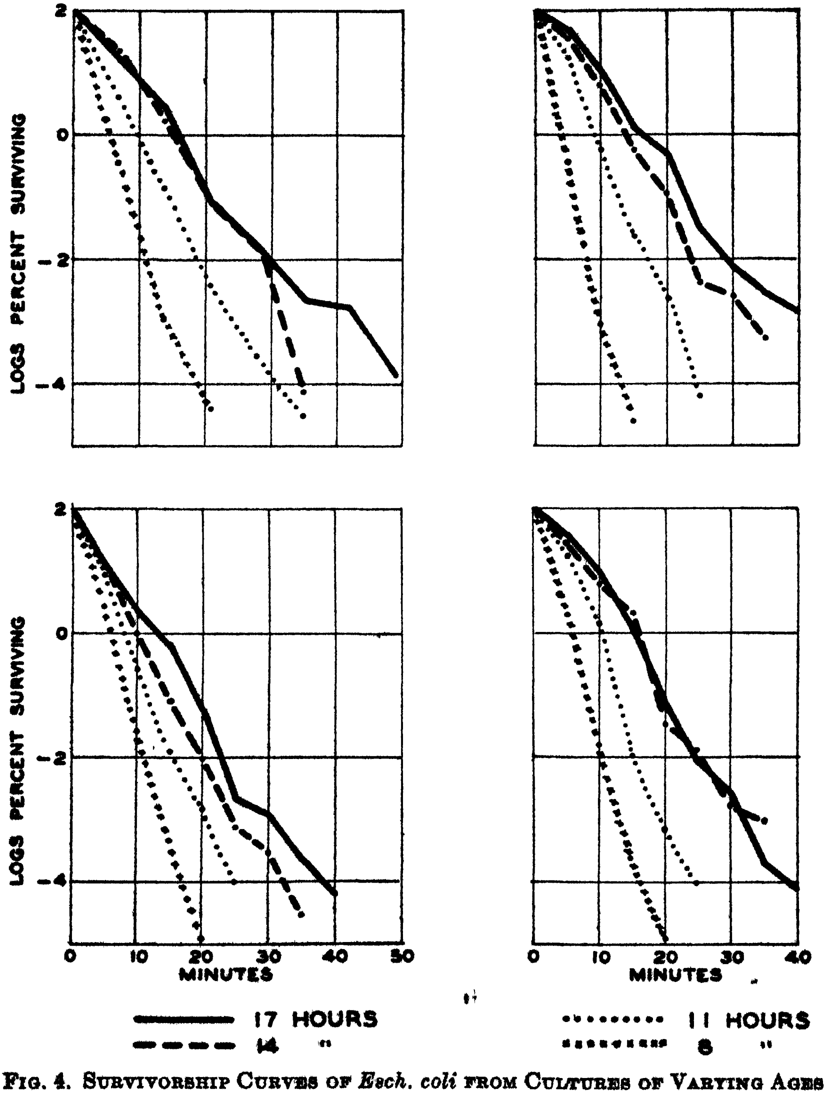

Fig. 4. Survivorship Curves of *Esch. coli* from Cultures of Varying Ages N/100 NaOH, 30°C.

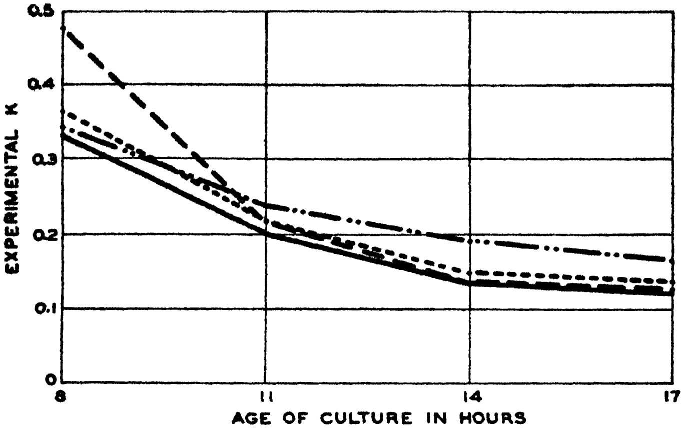

FIG. 5. RELATION OF AVERAGE EXPERIMENTAL *K* TO AGE OF CULTURE N/100 NaOH, 30°C.; four parallel experiments

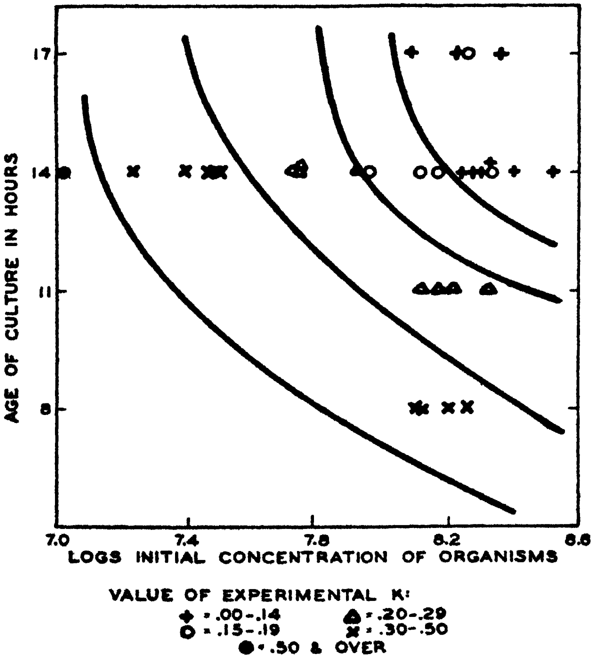

FIG. 6. RELATION BETWEEN AGE OF CULTURE, INITIAL CONCENTRATION OF ORGANISMS, AND AVERAGE EXPERIMENTAL *K*

from this area, the values of *K* progressively increase. The heavy lines on the chart have been drawn so as to separate the values of *K* into five major classes. The consistency of the results thus indicated is striking.

SUMMARY OF CONCLUSIONS

1. The studies here reported seem to indicate that, when vegetative cells (*Esch. coli*) are exposed to the toxic action of sodium hydroxide, reduction proceeds at a generally logarithmic rate and does not exhibit the increasing mortality rate with the progress of time which has been observed by Levine and his associates for spores. In our experiments, values for *K* (the coefficient for the rate of reduction per unit of time) were approximately constant throughout an experiment. We have estimated the ratio of *K* for the two time-halves of an experiment and find this ratio to average 0.97 for 109 experiments with NaOH, 1.17 for 16 experiments with a borate-buffer mixture and 0.96 for 26 experiments with hot water (50° and 55°). It seems possible that in the case of spore-destruction two distinct factors may be involved, one affecting penetration of a thick cell envelope and one toxicity; and that the different results observed with spores may be related to the former.

2. The rate of mortality in a cell population exposed to NaOH or to heat varies with at least four factors, as follows:

a. The age of the source-culture used for inoculation. Increasing the age of the source-culture from eight to eleven hours, decreases *K* by 42 per cent; a further increase to fourteen hours decreases it again by 30 per cent of the eleven-hour value; a further increase to seventeen hours decreases *K* again by 11 per cent of its fourteen-hour value. The *K* for a seventeen-hour culture is only a little over one third that for an eight-hour culture.

b. The concentration of the inoculum. Experiments starting with inocula of between one and fifty million cells per cubic centimeter show values of *K* from two to three times higher than those starting with inocula of over 100 million cells per cubic centimeter.

c. The intensity of the toxic agent. Doubling the concentration of NaOH increases the value of K about tenfold and increasing the temperature of water from 50° to 55° increases the value of K twenty to thirtyfold.

d. The temperature (when the toxic agent is a chemical one). At a given concentration of NaOH, an 8° rise in temperature increases K by from 35 to 109 per cent, the maximum effect appearing with low cell concentrations.

3. The influence of concentration of cells used in the inoculum may be attributed to the production by the cells of substances which form a protective zone about each cell and which will obviously remain more concentrated when in the neighborhood of similar zones surrounding adjacent cells. This is the phenomenon described by Shaughnessy and Winslow.

In the case of heat-killing we may perhaps assume that the zone of protective substances in the neighborhood of a cell reacts upon the cell wall to make it more resistant to the influence of heat.

It may be possible that the low resistance of cells taken from very young cultures is due to lack of power to form the protective substances in question; but it may equally well be due to some other factors associated with physiological youth.

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THE FERMENTATION OF ALPHA-METHYL-D-GLUCOSIDE BY MEMBERS OF THE COLI-AREOGENES GROUP

STEWART A. KOSER AND FELIX SAUNDERS

Departments of Hygiene and Bacteriology and of Physiological Chemistry, the University of Chicago

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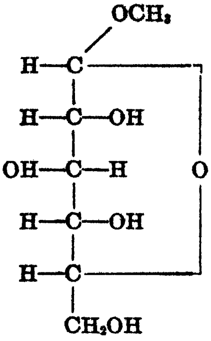
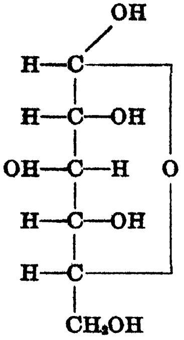
In the course of a study of the fermentation of certain substituted sugars by bacteria, it was found that alpha-methyl-d-glucoside was fermented with the production of acid and gas by several strains of *Bacterium aerogenes* but not by fecal *Bacterium coli*. Since the separation of these members of the coli-aerogenes group has assumed some interest, it was decided to extend this observation and to test a larger number of cultures.

For this purpose advantage was taken of a collection of *Bacterium aerogenes* and other related types which were isolated originally from soil and then kept as laboratory cultures for several years. In this collection of soil cultures were representatives both of the aerogenes section and of the soil colon cultures which are methyl-red positive, Voges-Proskauer negative and citrate positive (Koser, 1924). To complete the list of colon group cultures subjected to the test, a number of strains were isolated from normal fecal specimens of man and several of the lower animals: monkey, sheep, rabbit and guinea pig.

The entire series of coli-aerogenes cultures, including the freshly isolated fecal strains and the older soil strains, was then tested a number of times in broth containing alpha-methyl-d-glucoside. Finally, all cultures were subjected to the other tests recommended for differentiation of the members of this group of bacteria. Thus, the fermenatation of the glucoside was compared with the utilization of citrate, the fermentation of cellobiose, and the results of the methyl-red and Voges-Proskauer tests.

Alpha-methyl-d-glucoside differs from glucose in the substitution of a methyl radical for the hydrogen of the hydroxyl group attached to the number 1 carbon atom, as shown below. Two separate lots of the glucoside were used in our tests. One lot was secured from the Eastman Kodak Company, the other was prepared by one of us. Both lots gave identical results. Our preparation of the glucoside was accomplished by the usual method of refluxing glucose with methyl alcohol containing hydrogen chloride under anhydrous conditions (Patterson and Robertson, 1929). It was recrystallized until it no longer gave any reduction with Fehling's solution. The melting point was 168°C. (uncorrected).


Alpha-methyl-
d-glucoside


d-glucose

For fermentation tests, the glucoside was added in 0.5 or 1.0 per cent concentration to ordinary nutrient broth, pH 7.0. Brom-thymol-blue, or in some instances brom-cresol-purple, was added as indicator and the broth tubed with inner inverted fermentation vials. Sterilization was accomplished either by filtration or in the autoclave. In the earlier tests, when it was not known whether the glucoside could withstand autoclave sterilization, Seitz filters were used. A 10 per cent solution of the sugar in distilled water was filtered and then added aseptically to tubes of nutrient broth containing indicator. Later it was found that the results were the same after the usual period of autoclave sterilization. An incubation temperature of 30°C. was employed for most of the tests, though in one instance 37°C. was used with similar results.

In table 1 fermentation of the methyl glucoside is correlated with source of the cultures and with the other differential tests. Among the 103 fecal cultures, 3 were atypical in that they produced visible turbidity in the synthetic citrate medium and fermented cellobiose. The same 3 cultures fermented the glucoside. All of the other fecal cultures were consistently negative. Most of the 28 aerogenes types from soil presented a decided contrast to the fecal coli cultures. Twenty-three fermented the glucoside promptly, 4 produced slow fermentation and 1 gave a negative result. In the case of the methyl red +, citrate + types from soil (the "intermediate" strains) no uniform result was secured. Of a total of 25 strains, 5 produced prompt fermentation, 10 slow fermentation and the remaining 10 were completely negative throughout an incubation period of three weeks. It is evident that in the case of the so-called "intermediate" types, the fermentation of alpha-methyl-glucoside failed to show a correlation with source of the cultures. In separating the aerogenes

TABLE 1

Correlation of alpha-methyl-d-glucoside fermentation with source of cultures and with differential tests

SOURCE OF COLON GROUP CULTURES	NUMBER OF CULTURES	METHYL-RED TEST	VOGES-PROSKAUER TEST	UTILIZATION OF CITRATE	FERMENTATION OF CELLOBIOSE	FERMENTATION OF ALPHA-METHYL-D-GLUCOSIDE	SECTION OF GROUP
Feces (human, animal)	103	+ (103)	− (103)	− (100) + (3)	− (99) + (4)	− (100) + (3)	Mostly typical coli
Soil	28	− (27) + (1)*	+ (28)*	+ (28)	+ prompt (28)	+ prompt (23) + 4–6 days (4) − (1)	Aerogenes
Soil	25	+ (25)	− (25)	+ (25)	+ prompt (15) + 3 days (9) + 7 days (1)	+ prompt (5) + 3–4 days (6) + 6 days (4) − (10)	"Intermediate"

The number of cultures giving a positive or negative test is shown by the figures in parenthesis. Both acid and gas were produced as a result of fermentation of the alpha-methyl-d-glucoside and of cellobiose.

* One of the soil cultures was both methyl-red positive and Voges-Proskauer positive; otherwise an apparently typical aerogenes.

section from the typical fecal coli, however, the alpha-methylglucoside gave a satisfactory differentiation.

In other experiments it was found that the substitution of nutrient agar for broth as a base for the fermentation tests afforded just as reliable a separation of coli and aerogenes types. Gas production was sufficient to tear the agar medium and acid could be shown by the use of a suitable indicator. In some further tests it appeared that the glucoside was fairly stable. A batch of the glucoside broth was prepared, tubed, and sterilized in the autoclave at 15 pounds pressure for twenty minutes. Half of the lot was inoculated immediately with the entire series of coli-aerogenes cultures, while the other half was allowed to stand at room temperature for a month before inoculation. This was then inoculated with the same series of cultures and observed closely for any differences in fermentation, especially by the coli strains. No difference could be observed. Evidently glucoside broth may be kept for a considerable time after autoclave sterilization without detracting from the accuracy of subsequent fermentation tests.

Several preliminary experiments were made to determine the nature of the acids formed as a result of the glucoside fermentation by *Bacterium aerogenes*. In this case it seemed desirable to simplify the culture medium as far as possible and the synthetic citrate medium (Koser, 1924) was used, with the substitution of 1 per cent of glucoside for the citrate as the only source of carbon. In such a medium the aerogenes cultures developed more slowly than in nutrient broth, often requiring two to three days at 30°C. for the production of visible turbidity and acid. A representative *Bacterium aerogenes* culture was inoculated into 100 cc. amounts of the synthetic glucoside medium and subjected to chemical analysis after several different periods of incubation at 30°C.

Several cultures were distilled according to the Duclaux method, both with and without preliminary acidification. In no case could any steam-volatile acid be detected in the distillate. Other cultures were acidified and then extracted repeatedly with ether. No acid could be detected in the residue after the ether had been evaporated and qualitative tests for lactic acid were negative. Qualitative tests for acetic and tartaric acids were also negative. It is therefore concluded tentatively that the principal final metabolic product of *Bacterium aerogenes* in the synthetic glucoside medium is carbon dioxide.

SUMMARY

1. Typical *Bacterium coli* of fecal origin is unable to ferment alpha-methyl-d-glucoside.

2. Members of the aerogenes section usually ferment alpha-methyl-d-glucoside promptly with the production of acid and gas, though a few aerogenes strains produce a slow fermentation or give a negative result.

3. The fermentation of alpha-methyl-d-glucoside serves to separate the aerogenes section from fecal *Bacterium coli*. It is not so useful, however, for separation of the methyl-red positive soil types from the methyl-red positive fecal *Bacterium coli*. Both citrate and cellobiose are more reliable for this purpose.

4. Alpha-methyl-d-glucoside appears to be fairly stable. Nutrient broth containing 0.5 or 1.0 per cent of the glucoside can be sterilized in the autoclave and allowed to stand at room temperature for a month before inoculation without interfering with the results of the test.

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FACTORS INFLUENCING THE REDUCTION OF NITRATES AND NITRITES BY BACTERIA IN SEMISOLID MEDIA

C. E. ZoBELL

The George Williams Hooper Foundation, University of California, San Francisco, California

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Although the ability to reduce nitrates has been used for the identification of bacteria since the extensive investigations of Maassen (1902), the literature is still replete with inconsistencies and contradictory findings. This may be illustrated by observations on members of the Brucella group which have been reported. Eyre (1912), Duncan and Whitby (1930) and Bergey (1930) have described Brucella as being unable to reduce nitrates. On the other hand Evans (1918), Lustig and Vernoni (1928) and Topley and Wilson (1929) have asserted that nitrates are occasionally reduced. In the recent experiences of ZoBell and Meyer (1931) every strain of a collection of over 400 typical representatives of the genus Brucella vigorously reduced nitrates. Studies on this biochemical character in the "Salmonella" group are no less perplexing. Tittsler (1930) has summarized similar diametrically opposite descriptions in the literature concerning the ability of *Bact. pullorum* and *Bact. gallinarum* to reduce nitrates. Lack of agreement on the nitrate-reducing properties of many other common microörganisms has been reported.

In view of these incompatibilities a series of studies was undertaken to ascertain the cause of the incongruity. Either the testing procedures are at fault, thus leading to discordant results; or the ability of bacteria to reduce nitrates is inconstant; or certain strains of a given variety reduce nitrates while other strains lack such a mechanism. Regardless of which is true, little reliance can be placed upon the test for the differentiation of bacteria until these problems are solved. The present communication is primarily concerned with certain factors which influence the reduction of nitrates by microörganisms which have not been stressed by others.

EXPERIMENTAL

Approximately 600 cultures collected from various parts of the world were examined. Brucella and Salmonella predominated and two or more of 22 other common microörganisms were represented. A medium of the following composition was employed:

Bacto-peptone	2.0 grams
Beef-extract	1.0 gram
NaCl	3.0 grams
Agar	3.0 grams
KNO_3	1.0 gram
H_2O	1,000.0 cc.

The reaction was adjusted to pH 6.8. Preliminary experimentation indicated that the composition of the medium made very little difference except in nutrient properties as indicated by the multiplication rate. Practically any medium which supported a prolific growth was satisfactory. In exception to this, substances which alter the oxidation-reduction potential of the medium were important, as will be discussed later. The tubed media were uniformly inoculated with a 2 mm. loopful of a standardized suspension of the test organisms. Thus, each tube received approximately the same number of cells, objectionable clumps were avoided and the subsequent results were comparable. Nitrites were detected by the a-naphtholamine-sulfanilic acid method as described by Conn, *et al.* (1918) and the results were checked by the dimethyl-a-naphtholamine reagent recommended by Wallace and Neave (1927). In the absence of nitrites, nitrates were tested for by the zinc-reduction method: To the acidulated substrata containing the nitrite reagents about 20 mgm. of nitrate-nitrite-free zinc dust were added. The development of a pink color indicated the presence of nitrates. The importance of testing for nitrates when the nitrite test is negative merits emphasis. The procedure is advocated as an auxiliary test by the Standard Method (1930) when other tests have failed.

It was found that many of the Brucella and Salmonella types responded negatively when tested for the presence of nitrite after their growth in nitrate media. In routine practice this would usually be considered as evidence of their inability to reduce nitrates. However, an application of the zinc-reduction test showed that the nitrates were likewise absent from the substrata. In fact many of these cultures affected the disappearance of 0.1 per cent KNO_3 after two days of incubation. The only indication of the destruction of the nitrate molecule was the formation of gas by certain species. Table I illustrates typical findings.

TABLE 1

The presence of gas, nitrites and nitrates in media following the growth of bacteria for four days

BACTERIUM	DESIGNATION	GAS	NITRITES	NITRATES
Bact. sanguinarium	3139	−	−	−
	4187	−	−	+
Bact. pullorum	1618	−	+	++
	Utah	−	−	−
Br. abortus	7122	−	+++	++
	Th. 10	−	+++	++
Br. suis	Da 87	+	−	−
	H. F. 4	+	−	−
Ps. pyocyanea	U. C.	+++	−	−
Ps. denitrificans	Iowa	+++	−	−
B. subtilis	No. 2	−	−	++++
Blank	Control	−	−	++++

The fallacy of interpreting these data without information as to the presence of nitrates is self-evident. Even the minute amount of gas liberated was not perceptible except in semisolid media. Active nitrate-reducing organisms would have been considered as non-reducers. The Standard Method (1930) was designed to avoid this source of error by providing for the determination of nitrites on the first, second and fourth days. However, the careful scrutinization of a large number of cultures under different conditions has shown that many bacteria and particularly *suis* type Brucella and certain forms of Pseudomonas concurrently destroy the nitrites as they are formed. Only at irregular intervals does the concentration of nitrites become sufficiently high to give a positive test even with sensitive reagents. Some of the microörganisms cause the quantitative disappearance of the nitrite ions of 0.01 per cent KNO_2 more rapidly than they reduce an equivalent amount of nitrate. Bronfenbrenner and Schlesinger (1920) recognized this factor and suggested the use of a control containing 0.002 per cent KNO_2 or $NaNO_2$. The standard Method (1930) now recommends a medium with 2 p.p.m. KNO_2 as an alternative when other methods have yielded inconclusive findings in order to ascertain if the bacteria destroy nitrites. Apparently a large number of common bacteria are endowed with this ability, ZoBell and Meyer (1932) recently pointed out that this property which obtains in the Brucella organism to a marked extent has probably misled some investigators who have described members of the genus as non-reducers. Present experiences reveal that nearly all strains of *Bact. pullorum, Bact. gallinarum, Bact. sanguinarium, Bact. aerogenes* and *Bact. saccharolyte* can cause the destruction of as much as 0.01 per cent $NaNO_2$ in five days. It is not unlikely that this factor is largely responsible for contradictory reports concerning the first two Salmonellas referred to above. Some bacterial species were encountered which destroy nitrites without exhibiting any aptitude for reducing nitrates. Thus *B. fecalis-alcaligenes* and *Vibro metchinokovi* were found to be in this category. Therefore it appears advisable to include in the regularly prescribed tests on the "Descriptive Chart" the test for the ability of bacteria to break down nitrites. Aside from serving as an indispensable adjunct to the nitrate reduction test, it would also furnish valuable supplementary biochemical criteria for the characterization of microörganisms.

That the physical consistency of the media is a factor of prime importance in bacterial growth and metabolism was demonstrated by the preparation of media of three different viscosities. The first series consisted of firm gels containing 2.0 per cent agar, the second of gels of semisolid consistency with 0.3 per cent agar, while in the third no agar was used. Following the inoculation of these media with a uniform seeding of bacteria, evidence of multiplication first became perceptible in the semisolid series and continued to appear better in this medium. Some of the microaerophilic pathogens which were included in the experiment grew in the semisolid media but failed to proliferate in the liquid or solid media although the latter were identical in composition except for the gel structure. That the physical consistency of the media likewise influences the reducing activities of the bacteria was shown by the addition of redox indicators such as methylene blue and nitroanthraquinone in the proper dilution. The latter indicator was found to be preferable, since upon reduction the color passes through definite tinctorial stages from a colorless to a brownish-red compound. Invariably the dyes were reduced more rapidly in the semisolid substrata than in the solid or liquid media, probably due to the differential diffusion of atmospheric oxygen. The effect of the physical structure of the media on the reduction of nitrates by bacteria is illustrated by the data given in table 2. To demonstrate further the influence of the diffusion of atmospheric oxygen, the media were tubed so that the columns

TABLE 2

Nitrate reduction after two days' incubation of bacteria in media of different viscosities and depths

BACTERIUM	DESIGNATION	LIQUID		SEMISOLID		SOLID	
		5 mm.	50 mm.	5 mm.	50 mm.	5 mm.	50 mm.
Bact. pullorum	No. 9	−	++	+	+++	+	++
Bact. aertrycke	N. Y.	−	++	++	++++	++	+++
Bact. aerogenes	No. 2	++	++	++	+++	++	+++
Bact. paratyphosum	Pigeon	+	+	+	+++	++	+++
Strept. fecalis	Calif.	−	−	−	−	−	−
Br. suis	No. 80	++	++	+	++++	++	+++
Br. abortus	F 16	−	−	−	++	−	+
Br. melitensis	Africa 1	−	−	−	+	−	−

were 5 and 50 mm. deep respectively. Although the differences were not always clear-cut, conclusive tendencies were shown. Nitrate reduction was better when the columns were 50 mm. deep than when there were only 5 mm. of media in the tube; reduction in degree and in percentage of positive tubes was highest in semisolid media and lowest in liquid media. The advantages of the deeper columns and of the semisolid consistency are directly attributable to their influence on the oxidation-reduction potential of the media as will be discussed more fully in a later communication. The available evidence stresses the important influence of the oxidation-reduction potential on the multiplication of bacteria (Hewitt (1930)). It is to be expected that the oxidation-reduction potential has even a greater effect upon nitrate reduction, a reaction which depends upon the electron-escaping tendency or fugacity of the system. This is indicated by the studies of Korsakova (1929) which deal with the mechanism of the reduction of nitrates. The reports of Tiulpanova-Mosevich (1930) indicate that *Thiobacillus denitrificans* loses much of its nitrate-reducing ability when cultivated under aerobic conditions, probably due to the unfavorable oxidation-reduction potential activated by the oxygen.

DISCUSSION

For the routine examination of bacteria for their nitrate-nitrite splitting propensities the following procedure has yielded excellent results: The organisms are cultured in semisolid media containing 0.1 per cent KNO_3 and the necessary nutrient constituents to insure their multiplication. Instead of testing for the presence of nitrites on any prescribed day, the test is performed only after good growth has occurred and the time required for this will vary tremendously with different microörganisms. Either a-naphtholamine or dimethyl-a-naphtholamine with sulfanilic and acetic acids have been consistently satisfactory. If no color appears after a few minutes to indicate the presence of nitrites, about 20 mgm. of zinc dust are added directly to the substrata which already contains the nitrite reagents. Nitrates, if present, are thereupon shown by the development of the pink color.

Another medium which contains 0.002 per cent KNO_2 is always inoculated and tested to see if nitrites are destroyed.

Applying the principles described above, it has been found that the nitrate-reducing ability of microörganisms is a fairly constant characteristic. Results from the examination of the 600 cultures have been duplicated and repeated six months later with an accuracy approaching 100 per cent. Strains of given varieties collected from various parts of the world were quite alike in their ability or lack of ability to reduce nitrates. Thus, every strain of 420 Brucella reduced nitrates while others, like representatives of the sarcinae and certain streptococci, were uniformly negative. Therefore it seems that if precautionary measures are taken, absolute reliance can be placed on the nitrate tests for the characterization of bacteria. The ability of some microörganisms to destroy nitrites concomitantly as they are produced from nitrates is probably responsible for many contradictory findings and provisions must be made to avoid this source of error.

The superiority of semisolid media for the cultivation of bacteria recommends this physical consistency for the nitrate reduction test. Semisolid media combine most of the advantages of solid and liquid media and are for most purposes easier to handle than either of the latter. In summarizing some of the advantages of media containing small percentages of agar Hitchens (1921) pointed out that since such a medium retards the diffusion of oxygen, any degree of aerobiosis is procurable at some level of the medium column. Semisolid media permit the movement of motile organisms and the ready diffusion of nutrients and waste products. Hitchens found that many fastidious parasites which failed to multiply in liquid or solid media grew luxuriantly in semisolid media. Media which contain 0.2 to 0.3 per cent agar are particularly useful for studying the reduction of nitrates because in addition to enhancing multiplication they also favor the reducing activities of microörganisms. Many bacteria have been encountered which, although they fail perceptibly to reduce nitrates in liquid or solid media, react in semisolid media. Furthermore semisolid media are good indicators of gas production. Due to the viscosity which retards convection currents gas bubbles are visibly retained while in liquid or solid media minute amounts of the gas frequently escape unobserved. Also, the relationship of the organisms to atmospheric oxygen can be ascertained by noting the zone at which there is maximum proliferation. Aerobes will grow on or near the surface while facultatives and anaerobes will be found proportionately deeper. Thus it can be determined whether the organisms are able to derive their oxygen demands from the nitrate molecule as manifested by pseudo-anaerobic growth. All of these points furnish additional criteria for the identification of species. Finally, in testing for the presence of nitrates by the zinc method, the gel causes the zinc dust to be suspended throughout the medium and thus increases its sphere of action.

CONCLUSIONS

1. Approximately 600 strains of bacteria which have been examined were found to be constant in their ability to reduce nitrates.
2. Many species of microörganisms destroy nitrites as they are formed from nitrates so that nitrite tests are frequently negative although nitrates have been reduced. This property which obtains in the Brucella and in the Salmonella groups is in part responsible for the contradictory reports concerning their nitrate reducing ability.
3. It is recommended that a test for the presence of nitrates invariably be made in conjunction with the test for nitrites when the latter is negative.
4. The amplified test for the ability of bacteria to reduce nitrites is of sufficient import to warrant its inclusion in the Descriptive Chart.
5. The multifold advantages of semisolid media for the nitrate reduction test warrants its use as a Standard Method.

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STUDIES ON ROUGH AND SMOOTH VARIANTS OF SHIGELLA EQUIRULIS (B. NEPHRITIDIS-EQUI)[1]

PHILIP R. EDWARDS

Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington, Kentucky

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While engaged in the study of a number of cultures of *Shigella equirulis*,[2] extensive variation was noted in the cultural characters of this species. These changes were so pronounced that they were thought worthy of study and description. Other workers have noted variation in this species but no general agreement has been reached as to the extent and character of the changes. These writers have been interested primarily in mucoid and non-mucoid forms of the bacillus and have limited their statements concerning variation in the species to this property. In doing so they have failed to mention the most striking change taking place in the cultures, namely the presence of rough and smooth variations. Changes in the mucoid properties of the bacillus are very closely correlated with rough and smooth variation and it is possible to arrive at a better understanding of the changes if the two characters are considered together.

Lütje (1921, 1922) noted that the bacilli when originally isolated formed tenacious colonies but that after continued culture the organism produced a more moist, mucoid type of colony. These on further culture lost their tenacity entirely. Clarenburg

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[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

[2] The term, *Shigella equirulis*, has been applied to the species first called *B. nephritidis-equi* by Meyer and classified by Bergey (1930) as *Shigella viscosa*. The term *equirulis* is taken from *B. equirulis* of van Straaten, the first binomal applied to the species. This change is made since *Shigella viscosa* is not a valid term. For the complete list of synonyms applied to this species the reader is referred to the Kentucky Agricultural Experimental Station Bulletin 320 (1931).

(1924) observed that when the bacilli were cultivated artificially the mucoid character of the growth was gradually lost. Beller (1924) also noted the loss of mucoid properties on continued culture. Goerttler (1926) on the contrary found little if any deviation in the viscosity of 20 strains of the organism. He found the mucoid character so constantly that he states that when this property is lacking in a culture its identity must be questioned.

If the presence of rough and smooth variation and its relation to mucoid and non-mucoid forms is recognized it may be seen that variation in this species is more or less orderly and that the same changes take place in the majority of the strains of the organism. *S. equirulis* in most instances is isolated as a rough, mucoid type. As cultivation is continued the roughness of the colonies gradually disappears and eventually the bacillus produces smooth colonies. With this change in colony form the mucoid property is gradually lost. When first isolated the colonies are tough, rather dry, and very tenacious. This dry tenacious form gradually becomes more moist and stringy so that it will draw out in long threads when touched with a needle. This stage constitutes the smooth mucoid form. As artificial culture is continued the mucoid properties are lost until finally the culture becomes a smooth non-mucoid race. Roughness and tenacity are closely associated. In the isolation and cultivation of more than 100 strains of *S. equirulis* a rough culture has never been observed which did not produce tenacious colonies. Conversely, non-mucoid strains invariably form smooth colonies. Between these two extremes of rough mucoid and smooth non-mucoid there is the intermediate stage of smooth mucoid colony which is usually in a state of transition.

MATERIAL AND METHODS

The cultures used in the work are those described in previous papers (Edwards (1931, 1932)). Most of the work has been done with two strains, both isolated from foals dying at the age of twenty-four hours, and the variants derived from these strains. While the cultures used were not isolated from single cells they were derived from cultures which were apparently pure at the time they were isolated. These strains have been cultivated artificially from one to three years and during this period have been plated repeatedly. Some of the cultures, particularly the 2 strains 36 and 38 with which most of the work was done, have been plated 3 times each week for a period of two years. At no time has any evidence of contamination been observed. It is felt that there can be no reasonable doubt as to the purity of these cultures.

In the present work the morphology, cultural and biochemical characters, and serological properties of the rough and smooth forms have been examined. In addition the effect of certain environmental factors on the two forms and conditions which tend to bring about the conversion of one form into the other have been studied. Throughout the course of the work the substrates upon which the organisms have been cultivated have been kept as nearly constant as possible. The broth used to cultivate the organisms was composed of 1 per cent Bacto peptone and 0.3 per cent meat extract in tap water. The reaction was adjusted to pH 7.6. The agar used in transferring stock cultures and plating was prepared from fresh beef infusion and contained 1 per cent Bacto peptone and 2 per cent agar. The reaction was pH 7.6. Both the broth and agar were made up in large lots but since the work has covered a period of more than two years it has been necessary to use several lots of both mediums. Stock cultures were maintained on agar slants. They were transferred every sixth day, incubated at 37° for twenty-four hours and then held in the dark at room temperature.

MORPHOLOGY OF ROUGH AND SMOOTH FORMS

The morphology of *S. equirulis* is quite variable. This lack of uniformity, which has been emphasized by Goerttler (1926), extends to both the rough and smooth forms. However, from repeated examinations of a number of rough and smooth strains certain morphological differences are apparent in the two types. If agar cultures are examined at the age of eight to ten hours many unusual forms are found in both rough mucoid and smooth strains. Large yeast-like bodies bearing projections resembling buds are present. These extremely large forms take the ordinary stains very poorly. Long filamentous forms and streptococcus-like chains are found in both rough and smooth forms at this age. However, chains and filamentous forms occur much more commonly in the smooth than in the rough forms.

At fourteen to sixteen hours of age the difference in morphology of the cultures is much more pronounced. At this time the rough mucoid culture has assumed the morphological characters generally attributed to this species, the culture being almost entirely composed of short, oval rods. The smooth culture on the contrary will be found to abound in long filamentous forms streptococcus-like chains and very large poorly staining bacilli. After twenty-four hours' incubation the rough mucoid culture is composed entirely of short rods while filaments and chains are still numerous in the smooth culture. Streptococcus-like chains and filamentous forms are much more common at all ages in the smooth cultures than in the rough cultures.

Both the rough and smooth forms are non-motile. Capsule formation by either form has not been observed, although the rough mucoid form seems to produce an intercellular substance not found in the smooth form. This intercellular substance stains poorly or not at all by the ordinary methods of staining. However, the rough mucoid bacilli are apparently rather firmly imbedded in this substance, and when placed on a slide are held together in groups or masses.

CULTURAL CHARACTERS OF THE ROUGH AND SMOOTH FORMS

Agar colonies. Rough form: Well isolated agar colonies attain considerable size, 3 to 5 mm., in forty-eight hours. These colonies are raised, opaque, and extremely tenacious. At times the colonies are almost hemispherical. The edges of the colonies are undulate. The surface is extremely rough. When examined under the hand lens by reflected light they present a lobulated appearance, the small lobular projections appearing over the entire surface of the colony. Between the lobules deep crevices run irregularly over the surface. When viewed by transmitted light the roughened surface causes the colony to exhibit an irregular radially striated structure. With increasing age a clear smooth border zone may be extended from the colony.

Smooth form: The diameter of the smooth form is the same as, or slightly greater than, that of the rough form. Colonies are flat or very slightly raised, translucent, and non-mucoid. The edges of the colonies are entire. The surface is perfectly smooth. Viewed by transmitted light the colonies present a homogeneous structure. The colonies of the smooth form rather closely resemble those formed by members of the colon-typhoid group.

Agar slants. The growth of the two forms on agar slants possesses many of the characters of the agar colonies. The growth of the rough form is more opaque, roughened and tenacious than that of the smooth form.

Growth in broth. Rough form: The first growth to become apparent in broth cultures of the rough form is a collection of small masses of bacteria along the side of the tube. A few masses also may be observed floating on the surface of the liquid. As growth continues a light pellicle of uneven thickness is formed on the surface of the broth and the sides of the tube become covered with a deposit of the bacteria. A ropy sediment containing granules appears and eventually a diffuse growth occurs throughout the broth. Broth cultures of this form become very viscous.

Smooth form: The smooth form brings about an even clouding of the broth with little sediment and without pellicle formation.

BIOCHEMICAL CHARACTERS OF ROUGH AND SMOOTH FORMS

No differences have been demonstrated in the biochemical properties of the two forms, using the qualitative tests employed in these studies.

THE INFLUENCE OF CERTAIN ENVIRONMENTAL CONDITIONS ON ROUGH AND SMOOTH FORMS

Growth on agar. Growth on the surface of agar is favorable to the development of the smooth form. Rough cultures cultivated under the conditions outlined above for the maintenance of stock cultures soon begin to lose their roughness and tenacity.

After a few transfers the organisms are transformed to the smooth mucoid variety. They continue to become less mucoid until they reach the smooth non-mucoid form. The length of time required for this change to occur varies from four to five weeks to more than a year. A few cultures in our possession have reached the smooth mucoid state after a few transfers but on continued culture they have retained their mucoid characters. Other strains have changed from the rough mucoid form to the smooth non-mucoid state and then reverted to the mucoid form. However the majority of the strains have become non-mucoid and remained in this condition.

Growth in broth. The rough mucoid form is much more stable in broth than on the surface of agar. When rough mucoid cultures are transferred daily in 10 cc. of broth the smooth types are much slower in appearing than when the same strains are kept on agar. The ability of strains to maintain themselves in the rough mucoid form under these conditions varies. Strain 36 when treated in this manner gradually lost its roughness and was transformed to smooth non-mucoid. Strain 38, on the other hand, continued to produce mucoid colonies although the rough character disappeared. Continued culture in broth without transfer leads to a more rapid appearance of the smooth forms than does daily subculture in broth. *Shigella equirulis* does not remain viable in broth cultures for more than eighteen to twenty-one days. Within this time smooth colonies begin to appear. Daily transfer or continued culture of smooth forms in broth is not sufficient to restore smooth forms to the rough state. Some strains under these conditions will produce mucoid colonies but will not revert to the rough form.

Growth in culture filtrates. During the course of the work it was noted that when large numbers of colonies appeared on the plates of rough strains, the colonies were no longer rough but smooth. This suggested that the accumulation of metabolic products in cultures was responsible for the gradual change from rough to smooth in cultures of *S. equirulis.*

Rough and smooth forms of strain 36 were cultivated in broth and the cultures sterilized by filtration. The rough and smooth forms of this culture were inoculated into the filtrates of both the rough and smooth cultures. No growth took place in any of the tubes. The filtrates were then added to uninoculated broth in the amount of 25 per cent. Both the rough and smooth forms developed in the presence of 25 per cent of the filtrates. The cultures were transferred daily in these mediums for eighteen days and the twenty-four-hour broth cultures plated daily. Under these circumstances the presence of the culture filtrates apparently had no effect on the colony types produced by the organisms.

Growth at different temperatures. In order to determine the effect of temperature on the colony form of *S. equirulis* rough and smooth forms of strain 36 were grown in broth at 30°, 37° and 42°, The broth cultures were transferred daily for twenty-four days. Plates were streaked from the twenty-four-hour old broth cultures. The plates were incubated at the same temperature as the culture with which they were inoculated.

The results obtained with the rough form are as follows: The culture incubated at 30° continued to produce rough colonies throughout the course of the experiment. No smooth colonies appeared in this culture. Smooth colonies began to appear in small numbers in the culture incubated at 37° after the fourth transfer. These increased in number until the culture was producing only smooth mucoid colonies at the end of the experiment. Smooth colonies appeared in the second transfer of the culture kept at 42°. These increased in number and the cultures successively became less mucoid until at the end of the experiment only smooth non-mucoid colonies were present on the plates.

The smooth non-mucoid form of strain 36 when grown at 37° and 42° continued to produce smooth non-mucoid colonies. When grown at 30°C. the colonies became mucoid after the second transfer. At the end of the experiment the colonies were still smooth but very mucoid. It is evident that lowered temperatures favor the rough mucoid form.

Growth at different hydrogen ion concentrations. The rough form of strain 38 was planted in broth having the following hydrogen ion concentrations: pH 5.4, 6.0, 6.6, 7.2, 7.8, 8.0 and 8.4. The cultures were incubated at 37° and transferred daily.

Plates were streaked from the twenty-four-hour broth cultures on plates of corresponding reaction. The experiment was continued for eighteen days. No growth occurred in the broth having a reaction of pH 5.4. Growth was delayed and poor in the broth having a reaction of pH 6.0. On the corresponding plates the colonies that developed were small and few in number. After the third transfer these colonies became smooth and non-mucoid. The colonies developing on the pH 6.6 culture became smooth after the sixth transfer and toward the end of the experiment became non-mucoid. The remainder of the cultures produced rough mucoid colonies throughout the experiment. The cultures grown at pH 7.8 and 8.4 were extremely rough and tenacious.

It is apparent that an acid reaction favors the change from rough to smooth. It has been previously stated that the strain used in this experiment, no. 38, has less tendency to grow in the smooth form than the other cultures studied. Had another culture been used in this work it is probable that the changes observed would have been more extensive.

Growth in the presence of different amounts of normal horse serum. Plain broth and broth containing 10, 25, and 50 per cent normal horse serum were inoculated with a culture of strain 36 which was producing rough and smooth colonies in approximately equal numbers. The cultures were transferred daily and plates streaked from the twenty-four-hour broth cultures. The cultures were then corked to prevent evaporation and incubation continued. After varying periods these cultures were replated. Under these conditions normal horse serum exerted no influence on the type of colonies produced.

Growth in the presence of antiserum. Many workers have been successful in changing the colony types of organisms by cultivating them in their specific antiserums. In the present work the organisms were grown in plain broth, broth containing 25 per cent normal rabbit serum, and broth containing 25 per cent specific antiserum from rabbits. The cultures were transferred daily for twenty-four days and the twenty-four-hour broth cultures were streaked on plates. After plating the tubes were corked to prevent evaporation and incubation continued at 37°. After varying intervals the cultures were replated. The cultures used in this work were the rough and smooth forms of strains 36 and 38. The smooth form of strain 36 was derived from the rough form through growth in antiserum. The smooth form of strain 38 was isolated directly from the infected foal at post-mortem.

The rough form of strain 36 when grown in the presence of antiserum began to produce smooth non-mucoid colonies after the third transfer. During the course of the experiment the number of these smooth colonies increased until the culture was producing approximately 50 per cent smooth colonies. This change from rough mucoid to smooth non-mucoid took place without the appearance of the intermediate smooth mucoid form. The culture grown in 25 per cent normal rabbit serum continued to produce only rough colonies throughout the experiment.

The cultivation of the smooth form of strain 36 and the rough and smooth forms of strain 38 in antiserum failed to bring about any change in the character of the colonies produced.

Growth in large amounts of broth. A smooth non-mucoid culture of strain 36 was grown in 500 cc. quantities of broth and transferred daily. Plates were streaked from the twenty-four-hour cultures. Colonies arising from the first six transfers resembled the original cultures. On the seventh transfer the colonies were noticed to be more mucoid and a few were decidedly more opaque than the original. There were two colonies on this transfer whose surface was definitely roughened. The eighth and ninth transfers resembled the seventh. A roughened colony appearing on the ninth transfer was planted in 10 cc. of broth and plated after twenty-four hours. The colonies appearing on this plate were a mixture of rough and smooth. The roughest colony was again selected and planted in broth. By continuing this process of artificial selection a culture was obtained after six platings which had all the characters of the rough mucoid type.

Although the daily subculture in 500 cc. of broth was continued through 35 transfers no further change was noted in the culture. It continued to produce smooth mucoid colonies with the occasional appearance of a few slightly rough colonies. This experiment has been repeated with a number of strains and it has always been found necessary to resort to artificial selection to obtain a typical rough mucoid culture. The method outlined above is the only one by which we have been successful in obtaining rough cultures from strains which have become smooth.

SEROLOGICAL CHARACTERS OF ROUGH AND SMOOTH FORMS

As shown in a previous communication (1932) the serological characters of *S. equirulis* are not constant from strain to strain. A group of 40 cultures studied was found to be heterogeneous and few antigenic relationships could be established. It is evident then that any study of the antigenic relationships of the rough and smooth types must be carried out with the rough and smooth forms of the same strain or with strains known to be antigenically related. This has been done, using both the rough and smooth forms of strains 36 and 38 to prepare agglutinating serums. The results of the agglutination tests are given in table 1.

It can be seen that rough and smooth types are reciprocally agglutinated by rough and smooth antiserum. Strains 36 and 38 are not antigenically related and there is no cross agglutination by either their rough or smooth forms. Strain 34 was isolated as a rough strain but became smooth on continued culture. It is serologically identical with strain 36 and is agglutinated by both rough and smooth antiserums of that strain. Strain 6 was isolated as a smooth non-mucoid type and has never produced rough or mucoid colonies since its isolation. It is serologically identical with strain 38 and is agglutinated by both rough and smooth antiserums for that strain. No constant differences in the agglutinability of the rough and smooth forms were noted. Spontaneous agglutination occurred in certain strains but this had no relation to the rough or smooth type.

In testing the abilities of the variants to absorb agglutinins it was found that the smooth form of strain 36 was able to exhaust the rough antiserum of agglutinins for the rough form completely. Likewise the rough form was able to effect a complete removal of agglutinins for the smooth form from smooth antiserum. The same relationships were found to exist in the case of the rough and smooth forms of strain 38. Strain 34 (smooth) was able completely to remove agglutinins from 36 rough and 36 smooth antiserums. Strain 6 (smooth) was able to absorb agglutinins from both the rough and smooth antiserums of strain 38.

It is a significant fact, however, that much larger amounts of smooth bacilli were required to remove agglutinins from rough antiserum than were necessary when the rough form was used as the absorbing antigen. When smooth bacilli were used to absorb a rough antiserum the absorbing dose had to be increased 8 to 10 times in order to remove agglutinins completely. The explanation of this, we believe is included in the following paragraph.

TABLE 1

Agglutination of rough and smooth variants

ANTIGENS	SERUMS			
	36 rough	36 smooth	38 rough	38 smooth
36 rough	2,000	2,000	0	0
36 smooth	2,000	2,000	0	0
38 rough	0	0	2,000	2,000
38 smooth	0	0	2,000	2,000
34 smooth	2,000	2,000	0	0
6 smooth	0	0	2,000	2,000

0 indicates no agglutination at 1:20.

PRODUCTION OF SPECIFIC SUBSTANCES BY ROUGH AND SMOOTH FORMS

S. equirulis produces a soluble specific substance which can easily be detected in broth cultures and in the filtrates of autoclaved salined suspensions. Such filtrates serve well as antigens in the precipitin test. In order to determine the relative amounts of specific substances produced by rough and smooth forms saline suspensions of the rough and smooth forms of strain 36 were prepared. The turbidities were equalized and the suspensions autoclaved at 15 pounds pressure for fifteen minutes. The suspensions were cleared by Berkefeld filtration and used as antigens in precipitin tests. Both rough and smooth antiserums were used in these tests but since the results were nearly identical only the results with the rough antiserum are given. These are set forth in table 2.

It is evident that the rough mucoid culture produced a much larger amount of specific substance than the smooth non-mucoid culture. The filtrate from the rough culture caused marked precipitation when used in the amount of 0.01 cc. while the smooth form failed to cause precipitation when 0.1 cc. was used in the test. The fact that smooth non-mucoid cultures produce a greatly reduced amount of specific substance may account for the fact that increased amounts of smooth bacilli are necessary to absorb agglutinins from rough antiserum.

TABLE 2

Production of specific substance by rough and smooth forms

ANTIGENS	ANTIGEN AMOUNTS						
	1.0	0.5	0.3	0.1	0.05	0.01	Control
36 rough	++++	++++	++++	+++	+++	++	–
36 smooth	++++	+++	+	–	–	–	–

One-tenth cubic centimeter of no. 36 rough serum was used in all tests.

VIRULENCE OF ROUGH AND SMOOTH FORMS

The estimation of virulence of cultures of *S. equirulis* is rather difficult since it is non-pathogenic for laboratory animals. Adult horses are also quite resistant to the organism. Only young foals a few days of age possess a high degree of susceptibility. Since it has been impossible to obtain such foals to determine the virulence of the rough and smooth forms, it has been necessary for us to confine our observations to the production of local reactions in older horses. Such a method is admittedly unsatisfactory. The practice has been to inject subcutaneously the rough and smooth forms of the same strain into the same animal at different sites and to observe the effect produced. Under these circumstances no difference in the virulence of the two forms could be noticed. Both forms produced subcutaneous abscesses which ruptured and discharged a purulent exudate. After rupture the abscesses healed slowly.

We have noted, however, a marked difference in the virulence of recently isolated strains and strains which have been under artificial culture for some time. Recently isolated cultures not only produce local reactions in adult horses, but cause a general reaction with increased temperature, increased pulse rate, general depression and stiffness of the joints. As artificial culture is continued the organisms progressively lose virulence regardless of whether they are maintained in the rough or smooth form.

The fact that both the rough and smooth forms have been isolated in pure culture from the tissues of infected foals indicates that both are virulent for very young horses.

Variation in vivo. The fact that rough and smooth forms of *S. equirulis* are sometimes observed in the same foal indicates that variation may occur in the body of the infected individual. In an effort to determine whether such variation does occur pus was aspirated from all the abscesses produced in determining the virulence of the organisms. This pus was plated immediately and planted in broth and the broth cultures plated after twenty-four hours' incubation at 37°. No variation in the colony forms of the injected cultures were observed in these tests. Rough cultures were always recovered when rough cultures were injected. When smooth cultures were administered the colonies appearing on the plates were of the smooth variety. We have been unable to demonstrate any change in the character of the organisms in the body of the horse.

Isolation of smooth type from foals. The recognition of the smooth non-mucoid type of *S. equirulis* is of importance since it is sometimes isolated directly from the tissues of infected foals. It may be found associated with the rough mucoid type or may be present alone. Within the past four years 100 foals infected with *S. equirulis* have been examined. In 5 cases only the smooth form of the bacterium was present. In many other cases the smooth form was found associated with the rough form.

That these organisms were in reality *S. equirulis* can hardly be doubted. Although the heterogeneous antigenic characters of this species make its identification rather difficult, all of these forms possessed the cultural and biochemical properties generally attributed to this organism. All of these smooth forms lived for only short periods on the surface of agar, a character typical of *S. equirulis*. In addition some of the smooth forms have been shown to be closely related antigenically to certain rough mucoid strains. Some of the cultures which were smooth when isolated were transformed to the rough mucoid type through growth in large amounts of broth and selection of colonies. Other strains have remained smooth and non-mucoid in spite of attempts to cause them to become rough.

DISCUSSION

The question of existence of mucoid and non-mucoid strains of *S. equirulis* has been shown to be closely related to the phenomenon of rough and smooth variation. Rough cultures invariably have been found to be mucoid. Non-mucoid cultures always produce smooth colonies. In cultures which have been isolated for some time a smooth mucoid form is found. This intermediate stage between the two extremes may arise only in artificial cultures, at least it has never been observed in primary cultures from infective material. This form, if it ever occurs naturally in the animal body, is rare. The rough mucoid form seems to be the normal type of the organism, since it is isolated much more frequently than the smooth non-mucoid form.

The trend of variation in this species is from rough to smooth. While certain environmental conditions have been found to influence the type of colony produced, it has not been possible to regularly control variation in this species. The stability of the rough form varies greatly from strain to strain. Some strains, for example 38, can be maintained in the rough form without difficulty, while other cultures are very difficult to keep in the rough form. The stability of the rough form, also, may vary in the same strain at different periods. A culture which has been regularly producing rough colonies may suddenly begin to give rise to colonies which are less rough and more moist. The strain may then begin to produce extremely rough colonies again.

If any culture is to be maintained in the rough state indefinitely it is necessary to practice artificial selection constantly. Unless continued selection is resorted to the rough form cannot be long maintained. In this species the colonies possess all degrees of roughness and the change from rough to smooth and from mucoid to non-mucoid is a gradual one.

While some workers, notably Goerttler (1926), have denied that a non-mucoid form of *S. equirulis* exists, the results obtained in the serological studies should fix beyond doubt the identity of the smooth non-mucoid cultures being studied. It has been shown that these cultures were very closely related and capable of the mutual absorption of agglutinins. This fact, combined with the identity of their biochemical reactions, clearly indicates that they are members of the same species. Smooth non-mucoid forms have been found to produce a smaller amount of soluble specific substance than rough mucoid forms. However, we have never encountered a culture in which the production of specific substance was entirely suppressed.

SUMMARY

S. equirulis produces both rough and smooth colonies. The change from mucoid to non-mucoid is associated with a change of rough to smooth. Rough colonies are always mucoid while non-mucoid colonies are always smooth. There is a transitional stage of smooth mucoid. The trend of variation in artificial cultures is from rough to smooth. The rate at which the rough to smooth change occurs varies from strain to strain and may vary within the same strain at different periods. The rough form can be perpetuated in cultures only by continued selection. Growth in broth, incubation at lower temperatures, and an alkaline reaction favor the development of the rough form. Growth on agar, incubation at temperature above 37° and an acid reaction accelerate the change from rough to smooth.

The two forms are closely related serologically. The rough form produces a larger amount of specific substance than the smooth form. Both forms are isolated directly from the tissues of diseased foals, either alone or associated.

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PLATE I

Fig. 1. Rough Mucoid Colonies of *S. equirulis*, Forty-eight Hours, × 4

Fig. 2. Smooth Non-mucoid Colonies of *S. equirulis*, Seventy-two Hours, × 4

Fig. 3. Rough Mucoid and Smooth Mucoid Colonies of *S. equirulis*, Twenty-four Hours, × 4

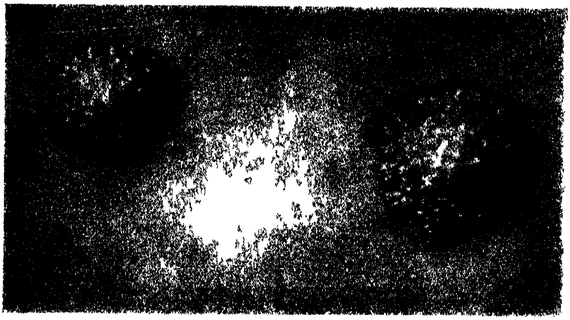

FIG 1

FIG 2

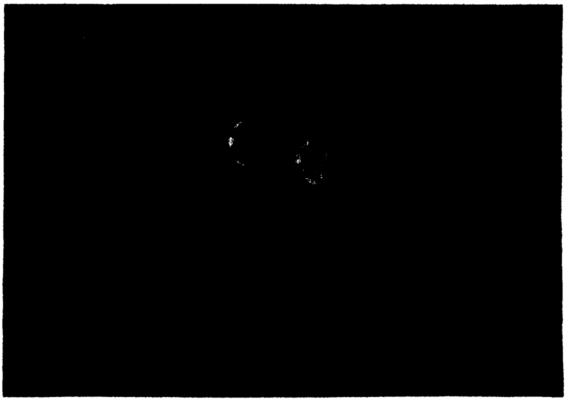

FIG 3

(Philip R Edwards Rough and smooth variants of S equirulis)

DISSOCIATION AND SENSITIVENESS TO NORMAL SERUM IN DYSENTERY BACILLI OF TYPE III

TH. THJØTTA AND ERIK WAALER

From the Bacteriological Laboratory of the Norwegian Army, Oslo

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Since Sonne (1915, a and b) described dysentery bacilli of type III in 1915 it has been known that these microbes might form peculiar colonies, which show a picture different from that presented by the colonies of the other types of dysentery bacilli. Sonne himself calls attention to the fact that this type is especially liable to form variants, which, he supposes, is the reason why this type so often shows irregularities of agglutination. Thjøtta (1917, 1918, 1919) describes the colonies of type III as being somewhat larger than those of the other types, a little denser and showing a peculiar crenated irregular edge. He studied these colonies thoroughly and found that they must be considered typical of type III, since they were always found in this type and never in the other types of dysentery bacilli. He also found that type III was characteristic in so far that the strains of this type all agglutinated in fine granules and showed a low titre as compared to the other types (i.e., type I, the Shiga type and II, the Flexner types). Further, the strains of type III showed a peculiar sensitiveness against normal active serum, which was only occasionally found in the other types. Other investigators, who have studied the colonies of type III are Elkeles and Schneider (1927). These authors find round regular colonies and irregular colonies belonging to type III. They consider the irregular ones as degenerated and claim that the round ones show the connection between this type (type III) and the other types of dysentery bacilli. Ørskov and Larsen (1925) also discuss variants of the colonies of type III. They find two main types, one which forms round, regular colonies and one, irregular colonies. These two types, however, form subtypes that behave differently, fermentatively and serologically. Ørskov and Larsen think it possible that the different colonies may be produced through the action of a bacteriophage. Koser and Styron (1930) in their study of dissociation of the colonies of type III use the terms smooth and rough, respectively for the regular and the irregular colonies, and thus place the colonies, where they obviously belong, among the dissociation phenomena of dysentery bacilli of type III.

MATERIAL AND METHODS

Our intention in this study has been first to find out whether or not the colonies of type III occur both as the smooth and as the rough variant in the very first cultures from the patient, and then to study the serological reactions of the two variants, and especially the bactericidal reaction of both types. To obtain fresh material we had to wait until occasion placed in our hands some cases of dysentery, caused by type III. The occasion came during the spring of 1931, when we had the luck of obtaining five specimens of stools within short intervals, all containing dysentery bacilli of type III.

The technique used in the examination of dysentery stools is simple. A small portion of mucus is washed in normal saline and streaked on a series of litmus-lactose-agar plates, which are examined after twenty-four hours in the incubator. All suspicious colonies are examined for agglutination in a serum belonging to type III. The positive ones are studied as to their morphological characters, picked and planted on new plates for further examination.

In four out of the five original stool cultures we found two kinds of colonies, which were lactose-negative and agglutinated in an old specimen of serum, produced with a very old strain belonging to type III. In the fifth culture, we found only one kind, namely a round, shiny colony, which however, on the first subculture, gave a growth consisting of the same two kinds, met with in the first culture from the four other specimens of stool.

Thus it may be said that our five cultures of dysentery bacilli isolated directly from the patient, suffering from typical dysentery, contained two kinds of colonies, and these two different colonies showed both the characteristics of dysentery bacilli and especially those of type III, which it will be unnecessary to restate in this paper.

One of these colonies was of the same shape as the ordinary colonies of dysentery type I and II, i.e., it was a round domed colony with a shiny surface and a regular edge. Morphologically it was a typical "smooth" colony. The other colony was larger than the first one, flat with a crenated irregular edge and a rather dry and slightly verrucose surface. This colony, which was the colony described as the typical one in dysentery III consequently was a typical "rough" colony. On further cultivation through several subcultures, the rough colony always held its type unchanged, while the smooth one in each culture gave growth of both smooth and rough colonies, but always so that the rough colonies were more abundant than the smooth ones; gradually the smooth culture died out, leaving only rough colonies, if no especial precautions were taken.

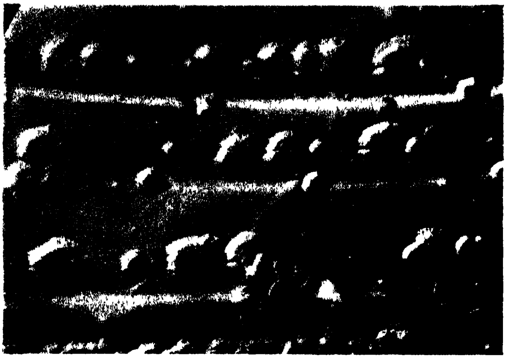

FIG. 1. S AND R COLONIES OF TYPE III ON LITMUS-LACTOSE AGAR, NATURAL SIZE

Morphologically there was no doubt as to the smooth and rough characters of the two different colonies. The other characteristics of these two types of colonies also were typical. An emulsion of the smooth colony in normale saline was stable for days, while the emulsion of the rough type sedimented within twenty-four hours. The smooth type agglutinated in coarse flakes, while the other type was agglutinated in fine dry granules.

We find it consequently justified to claim that dysentery of type III will produce both smooth and rough colonies when ob-

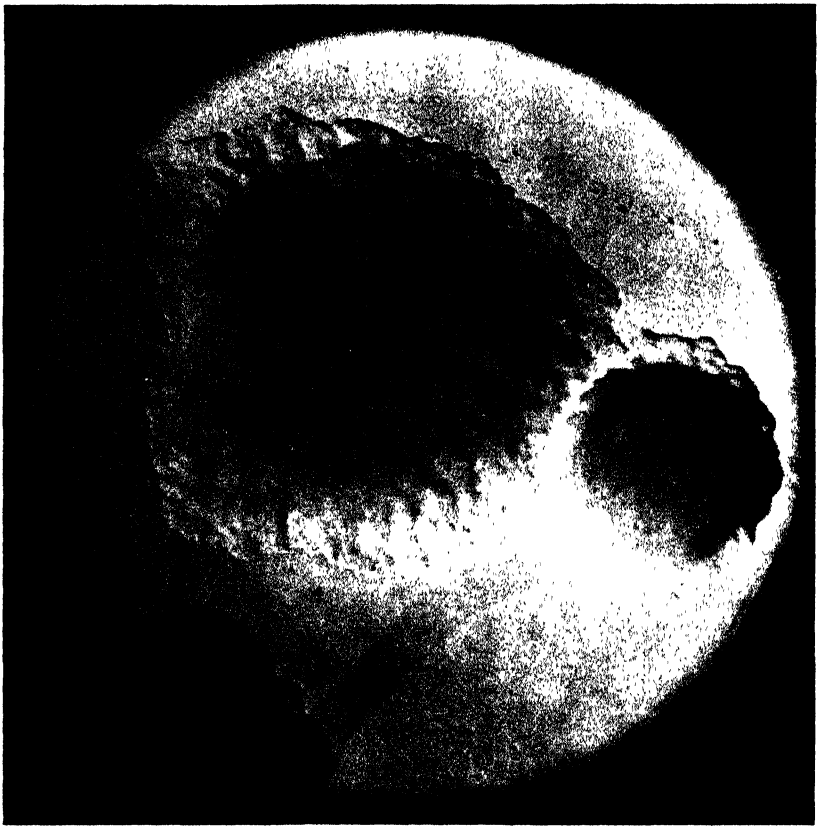

FIG 2. S AND R COLONY, ENLARGED 15.1

tained directly from the patient in the first or second generation. The smooth colony is very unstable and soon gives way for the rough colony, which will be found in all laboratory strains of this dysentery bacillus. The rough type of colonies is that claimed by Thjøtta in 1920 to be typical for the third type of dysentery bacilli.

Between the two pure smooth and rough types of colonies several intermediate types are found, which are almost impossible to place at present. Only two will be mentioned in this paper, first the colony described by Thjøtta as looking like a bunch of hair. This colony may very often be seen in cultures containing both smooth and rough colonies. The peculiar feature of this colony is that one side shows pure smooth characters, while the other side shows a rough picture. The rough part of the colony is often very small, so that it looks like a rough sector growing out of the body of the smooth colony. This colony is

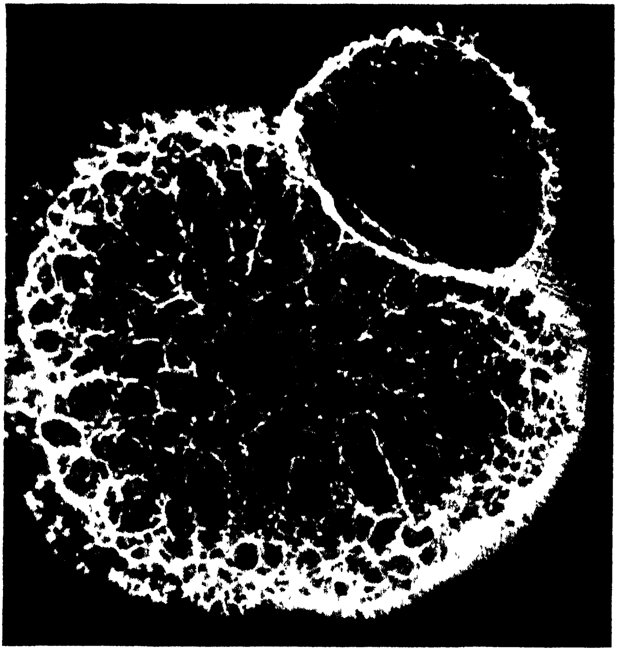

FIG 3 S AND R COLONY IN DARK FIELD ILLUMINATION, ENLARGED 15 1

Picture shows the colonies to consist of larger and smaller "islands" of bacterial conglomerates, which build the colony The islands are much larger and coarser in the large (rough) colony, than in the small (smooth) colony only a stage on the road from the smooth to the rough colony. The other type of colony we will mention is a colony derived from cultures of R in 1 per cent glucose broth after the technique of Koser and Styron. These authors produced S colonies from R through the procedure of growing the R type in this medium for several generations. We also tried this technique. We put an R type through 50 generations of glucose broth, spreading it after each tenth generation to study the colonies, which developed. After 50 generations we obtained a colony that was distinctly different from the R type and so much like the S type, that we at first believed it to be a real S colony. A closer examination showed, however, some characteristics that were somewhat different from the S type. The colonies were very little smaller than the ordinary S colonies, and likewise somewhat denser. The surface was, however, completely smooth and shiny, and the emulsion in saline was as stable as that of the real S type. Serologically this type was, however, quite different from the ordinary S, in fact showed characters identical to the R type. We therefore called this type "pseudosmooth." This pseudosmooth type seems to be a quite stable type of its own, but, as will be seen later, it must obviously be classed with the R type.

SEROLOGICAL CHARACTERS

1. *Agglutination*

During the routine investigation of cultures from dysentery stools we always use a preliminary agglutination test of the colonies met with in the different type sera on the surface of a slide. Our test serum from type III was produced with an old strain in its R type. It has already been mentioned that we obtained both S and R types of colonies in all of the primary cultures with the exception of one. All these cultures, both of the S and the R type, gave a positive agglutination test on the slide, and the S type gave a coarser and more distinct agglutination than the R. One of our strains was then injected into rabbits both in its S and its R type and immune sera were produced. The titer of the S serum was 1:1280 for all S types, while the titer of the R serum was 1:640 for all R strains with the exception of one that showed 1:320. The type of agglutination was the typical coarse agglutination in the S type, while the R type showed the fine granular agglutination.

TABLE 1

Agglutination tests in serum from type III, produced in rabbits with the S and R type of the bacillus

STRAINS	DILUTION OF SERUM								
	10	20	40	80	160	320	640	1,280	2,560
					S serum				
Type I:									
Shiga	−	−	−	−	−	−	−	−	−
Type II:									
F 105	−	−	−	−	−	−	−	−	−
"D"	+	+	−	−	−	−	−	−	−
"Skien"	+	+	−	−	−	−	−	−	−
Type III:									
S	+++	+++	+++	+++	+++	+++	++	+	−
R	+	+	+	+	−	−	−	−	−
"Pseudo S"	+	+	+	+	+	−	−	−	−
					R serum				
Type I:									
Shiga	−	−	−	−	−	−	−	−	−
Type II:									
F 105	+	+	+	−	−	−	−	−	−
"D"	+	+	−	−	−	−	−	−	−
"Skien"	+	+	−	−	−	−	−	−	−
Type III:									
S	++	++	+	−	−	−	−	−	−
R	+	+	+	+	+	+	+	−	−
"Pseudo S"	+	+	+	+	+	+	+	+	−

+++, agglutination with coarse flakes; ++, agglutination with less coarse flakes; +, agglutination with fine granules; −, no agglutination.

A serological comparison between the two variants of type III and the other types of dysentery bacilli is seen in table 1. Type I (the Shiga type) does not show any agglutination either in S or in R serum from type III. From type II (the Flexner type) three different strains are tested. No specific agglutination is observed. Two strains show a slight normal agglutination in both the S and the R serum. One strain, however, agglutinates only in the R serum and to the titer 1:40. This strain happened to be a rough type II strain. It may possibly be that the rough character of this strain makes it show some slight agglutination in the R serum from type III without reaching the titer of the homologous strain.

Thus, we find that the pure S and R strains from type III show the typical agglutinative reactions of the variants, and we also find that type III is distinct from the other types of dysentery bacilli whether the serum is produced with an S form or with an R form of the same strain.

The agglutinative reactions of the intermediate types between S and R will not be mentioned in this paper with the exception of the type called pseudosmooth. This type agglutinated to the titer limit of the R serum and with the same type of agglutination, while it did not reach more than one-eighth of the titer of the S serum. According to its agglutination this type must be regarded as an R type.

2. Sensitiveness against normal serum

In his work on the serological characters of dysentery bacilli of type III Thjøtta has called attention to the great sensitiveness of type III in normal, active sera of human or animal origin. This character seemed to be much more predominant in this type than in any of the other types. As we know that Thjøtta in his study was dealing with the rough type of the said bacillus, it will be of interest to study the bactericidal sensitiveness in normal active sera both of the R and of the S type and possibly find some difference between the two variants in this respect.

By the term "total bactericidal test" we understand the phenomenon which occurs when living bacteria added to solutions of fresh, active and normal sera are inhibited in their growth either partially or completely, so that cultures from the emulsion of bacteria and serum in different dilutions will yield either a slight growth or no growth at all. The technique of these tests is simple. In a series of test tubes the serum to be examined is diluted in a geometrical series from an upper limit, e.g., 0.1 or 0.05 cc. The dilution is made in normal saline and two drops of broth are added to each tube for better growth. The total amount of fluid is 0.5 cc. and consequently, 0.5 cc. of serum used in some titrations represents concentrated serum in these tests. The amount of living bacteria used has been 1:8000 of a loopful of growth from an agar slant of the microbe to be examined.

After titration of serum and addition of bacteria, the tubes are placed in the incubator for a convenient amount of hours, as a rule for three hours. The tubes are now taken out and streaks are made from each tube on the surface of agar plates. Six tubes can be spread on one plate, using the six sectors made by drawing on the bottom plate with a glass pencil (see figs. 4 and 5). After the seeding from each tube, they are put into the incubator again for further growth until the next day. At this time the colonies are counted on the agar plates and the growth can be seen as cloudiness in the original test tubes. The growth can thus be compared and checked.

An actual instance of a bactericidal test using this technique is the following:

Type III, Fredrikstad, rough

ACTIVE SERUM FROM GUINEA PIG	COLONIES	GROWTH IN TUBES
cc.		
0.05	1	0
0.025	6	0
0.0125	5	0
0.0063	15	0
0.0032	100	++
0.0016	∞	++
0 control	∞	++

A number of tests are made with this technique. Table 2 shows the result for S and R from three different strains belonging to type III and for two R strains also belonging to the same type. It will be seen that the R type is regularly sensitive to the bactericidal powers of normal serum, while the S type is resistant.

TABLE 2

Bactericidal tests in guinea pig serum with different strains of dysentery bacilli of type III

DILUTION OF SERUM	STRAIN 1		STRAIN 2		STRAIN 3		STRAIN 4	STRAIN 5
	S	R	S	R	S	R	R	R
cc.								
0.05	M +	60 −	M +	6 −	M	0	0 −	1 −
0.025	M +	31 −	M +	2 −	M	40	7 −	6 −
0.0125	M +	12 −	M +	13 −	M	23	4 −	5 −
0.0063	M +	M +	M +	35 −	M	50	25 −	15 −
0.0032	M +	M +	M +	M +	M	M	12 +	100 +
0.0016	M +	M +	M +	M +	M	M	M +	M +
0 control	M +	M +	M +	M +	M	M	M +	M +

S, smooth strain; R, rough strain; M, a growth of more than one thousand (Mille) colonies in the surface seedings from the tubes after three hours' contact with serum; +, visible growth in the original tubes after twenty-four hours' incubation; −, no visible growth in tubes. Figures 0, 1, etc., signify number of colonies in surface seedings.

TABLE 3

Bactericidal tests in guinea pig serum with the S and R type of strain I of dysentery bacilli of type III

SERUM	SEEDINGS AFTER HOURS OF CONTACT BETWEEN SERUM AND MICROBE								
	0	1	2	3	4	5	6	7	24
Test with the S type									
cc.									
0.5	M	200	100	50	3	3	0	1	M
0.4	M	200	100	100	100	100	100	200	M
0.3	M	200	100	100	200	500	500	M	M
0.2	M	500	500	500	500	M	M	M	M
0.1	M	M	M	M	M	M	M	M	M
Test with the R type									
0.05	M	1	0	0	0	0	0	0	0
0.025	M	25	0	0	0	0	0	0	0
0.0125	M	M	1	0	0	0	0	0	0
0.0063	M	M	M	9	1	1	1	1	1
0.0032	M	M	M	M	M	M	M	M	M

Signs as in table 2.

In Table 3 the bactericidal effect of normal guinea pig serum is shown both for the S and the R type from hour to hour for the seven hours following the start of the experiment and after twenty-four hours. The experiment with the S type starts in concentrated serum and goes down to a dilution of 1:5. It will be observed that the concentrated serum inhibits the growth of the microbes, but cannot annihilate them completely, as the twenty-four-hour test shows full growth. The experiment with the R type of this same strain of microbes starts with a dilution of serum of 1:10 and goes down to 1:160. The table shows that a diminution of bacteria is observed after one hour's stay in the

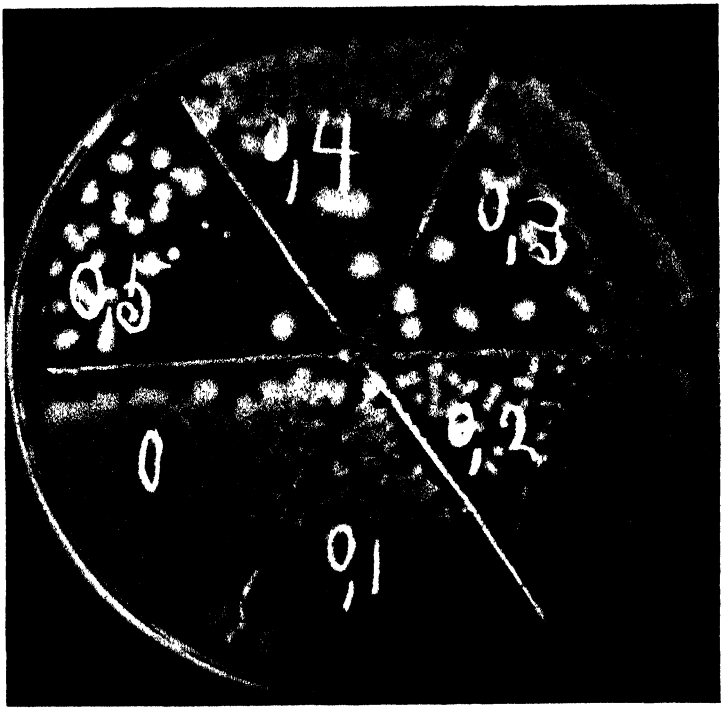

Fig 4 Bactericidal Test in Guinea Pig Serum with the S Type. Doses of Serum Written on the Different Sectors Seedings After Four Hours Contact in Incubator between Serum and Bacteria Natural Size

incubator. A dilution of 1:80 is capable of producing a permanent inhibition of growth, i.e., a complete dissolution of the bacteria.

Table 4 shows the bactericidal effect of two normal rabbit sera (A) against the S and the R type of the same strain of type III

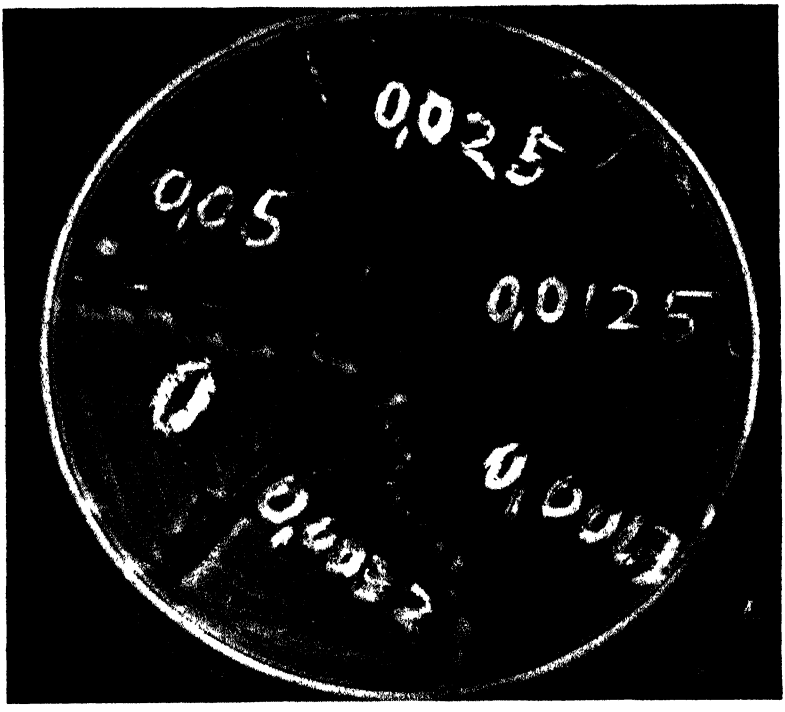

FIG. 5. BACTERICIDAL TEST WITH R TYPE IN SAME SERUM. SEEDINGS AFTER FOUR HOURS' CONTACT. NATURAL SIZE

and the test after immunization of the rabbits with the same strains (B).

It will be seen that the sera from both animals dissolve the R type and are ineffective against the S type. The B part of this experiment shows the complete ineffectiveness of both sera against both types after the immunization of the animals. The reason for this peculiar behaviour of the sera is the inhibition of the bactericidal effect due to the so-called Neisser-Wechsberg phenomenon. The inhibition of Neisser-Wechsberg is so strongly pronounced in these sera, that it descends farther down in the titrations of the sera than does the total bactericidal effect. When no foreign complement is added to the tubes, as is the case in our tests, one will never see any bactericidal effect of an immune serum, if the Neisser-Wechsberg phenomenon is strong enough.

TABLE 4

Bactericidal tests in active rabbit sera with the S and R type of strain I of dysentery bacilli of type III

A. Before immunization of the animals

SERUM	RABBIT 70				RABBIT 71			
	S		R		S		R	
cc.								
0.05	M	+	2	–	M	+	12	–
0 025	M	+	2	–	M	+	3	–
0 0125	M	+	9	+	M	+	2	+
0 0063	M	+	M	+	M	+	100	+
0 0032	M	+	M	+	M	+	M	+
Control	M	+	M	+	M	+	M	+

B. After immunization of the animals

SERUM	RABBIT 70 IMMUNIZED AGAINST THE R TYPE				RABBIT 71, IMMUNIZED AGAINST THE S TYPE			
	S		R		S		R	
cc.								
0 05	M	+	M	+	M	+	M	+
0.025	M	+	M	+	M	+	M	+
0.0125	M	+	M	+	M	+	M	+
0.0063	M	+	M	+	M	+	M	+
0.0032	M	+	M	+	M	+	M	+
Control	M	+	M	+	M	+	M	+

Signs as in table 2.

This phenomenon is of some theoretical interest. It shows, that the lytic agent active in our tests is due to the serum itself. If the R type had been a lysogenic colony harbouring a lytic principle due to a bacteriophage it cannot be explained why this principle should not be active in an immune serum, but fully active before treatment of the animal.

Besides guinea pig and rabbit serum some sera from human origin have been tried as to their bactericidal activity against the S and R type of type III. The result of these tests were the same as those dealt with.

It seems after these results that we are justified in concluding that dysentery bacilli of type III are resistant to the bactericidal action of normal serum in their S type and sensitive in their R type. This is obviously the reason why Thjøtta in his former work on this subject claimed the sensitiveness against serum as a special characteristic of type III. He always worked with the R type, since this type regularly developed in the first generations of this bacillus. It is of considerable interest, that dysentery bacilli of type III call forth the least dangerous clinical type of dysentery, and that, especially, this type so easily dissociates into the R type, which again is so easily dissolved by normal serum.

If the reactions dealt with hold true for the S and R type of type III, it should be natural, that the bactericidal test might be of practical use in purifying a mixed culture of S and R. This also is the case. A culture of this kind will give a very pure S culture if treated with active normal serum, and the best method of keeping an S type in pure shape is putting it through a bactericidal test now and then. This being the case, one would possibly be justified in claiming, that the bactericidal test should be of practical use in the study of atypical dissociations of dysentery bacilli of type III. The before mentioned pseudosmooth colonies have thus been subjected to several bactericidal tests and always found to be very sensitive to the normal serum. In this respect this type accordingly must be classed as an R type, in spite of the smooth and shiny surface and the stable emulsion.

It will of course be of great interest to study other bacteria in their S and R type as to the bactericidal effect of normal sera upon the two different types. We have been able to study an old strain of paratyphosus B bacilli in this respect. This strain yielded both S and R colonies, and table 5 shows, that the S type is resistant in rabbit and guinea pigs serum, while the R type is sensitive.

An old strain of *B. typhosus* and one strain of paratyphosus A gave growth only of R types, and these were very sensitive in both cases. Another strain of *B. typhosus* gave growth of a few R like colonies besides the regular S colonies. In the bactericidal test with these R like colonies a full growth came forth, and all colonies were now typical S. This experiment shows how the bactericidal test in this case settled the question of the nature of some atypical colonies.

The behaviour of strains belonging to dysentery bacilli of type I and II (the Shiga and Flexner types) in relation to the bactericidal test has not as yet been thoroughly studied, because we have not been in the possession of fresh newly isolated strains of these types. These types will be studied as soon as typical strains are obtained.

TABLE 5

Bactericidal tests in guinea pig and rabbits serum with S and R types of paratyphosus B

SERUM	GUINEA PIG SERUM				RABBIT SERUM			
	R		S		R		S	
cc								
0 05	14	−	M	+	1	−	M	+
0.025	30	−	M	+	100	−	M	+
0 0125	40	−	M	+	M	+	M	+
0.0063	40	+	M	+	M	+	M	+
0.0032	80	+	M	+	M	+	M	+
0 0016	M	+	M	+	M	+	M	+
Control	M	+	M	+	M	+	M	+

Signs as in table 2.

CONCLUSIONS

1. Dysentery bacilli of type III produce both S and R colonies in the first culture from the stool. The S colonies are very unstable, while the R colonies are found in all laboratory strains of this type of dysentery bacilli.
2. The colony described earlier by one of us as the typical type III colony is in reality the R type of this bacillus.

3. The reason for the low titres of agglutination both in sera from patients and from animals after immunization formerly described is that the R type of the bacillus and not the S type has been used.

4. The reason why dysentery bacilli of type III have been found to be exceptionally sensitive against normal active serum, is to be found in the fact that the R type of this microbe is very sensitive to normal serum. This is not the case with the S type of the same microbe, which is resistant against the same serum, that will dissolve the R type. As the R type always has been dealt with in bactericidal tests, the characters of this type have erroneously been thought to be those of the species.

5. There is some reason to believe that the R type of Gram-negative intestinal rods will prove sensitive against serum, while the S type will be resistant.

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A NOTE CONCERNING THE EFFECT OF A SPECIFIC ENVIRONMENT ON THE CHARACTERISTICS AND VIABILITY OF SEVERAL STRAINS OF AEROBACTER AEROGENES AND ESCHERICHIA COLI

WALTER L. KULP

Connecticut Agricultural College, Department of Bacteriology

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INTRODUCTION

The question of the stability of bacterial characteristics has received considerable attention since the work of Löhnis and Smith in 1916. The phenomenon of dissociation as shown by Hadley and others, and the evidences of definite life cycles as demonstrated by Löhnis and Smith, Mellon, Henrici and others have completely revolutionized the bacteriologist's ideas regarding the constancy of species.

This paper is a report of a research extending over a period of three and one-half years, and deals with the effects of a limited specific environment on the characteristics of several strains of *Aerobacter aerogenes* and *Escherichia coli*, two species which have many similar characteristics.

A study of the literature indicates the general opinion that *Aerob. aerogenes* and *Esch. coli* have the following differential characteristics:

	AEROB. AEROGENES	ESCH. COLI
Voges-Proskauer reaction	+	−
Methyl-red test	−	+

In the determination of the stability of strain characteristics, the following factors have been considered:

Morphology
Cultural characteristics
Gram staining
Lactose fermentation
Indol production
Voges-Proskauer reaction
Methyl-red reaction
Development in citrate broth
Action in nutrient gelatin
Growth in uric acid broth

These appear to be the basic factors to be considered in placing any strain in either the Aerobacter or Escherichia genus. For this reason no attention has been paid to any other characteristics of the cultures which have been studied.

EXPERIMENTAL PLAN

The cultures of *Esch. coli* and *Aerob. aerogenes* employed were purified by growing them in lactose broth, then by streaking an Endo plate and culturing a selected colony. This process was repeated three times before the culture was considered pure.

Each apparatus containing the limited specific environment was prepared in the following manner:

A small hole was blown into the bottom of a $\frac{3}{4}$- by 6-inch test tube. This tube was filled about half full with coarsely-sieved garden soil and the mouth was plugged with cotton. After a strip of cotton had been wrapped around its middle, this cotton-wrapped tube was inserted into a large-mouthed bottle in such a manner that the tube was held suspended in the bottle. The cotton wrapped around the middle made a tight plug for the bottle mouth, so that no contamination could enter. This entire set-up was then held in an autoclave for thirty minutes at 121°C.

The soil tubes were inoculated by the addition of 1 cc. of a twenty-four-hour nutrient broth culture. At rather infrequent intervals, sometimes as great as three months apart, the soil culture was thoroughly soaked with sterilized tap water. The hole in the bottom of the test tube permitted the drainage of excess water. On the day following one of these moistening operations a large loopful of the moist soil was transferred to lactose broth. When growth appeared in this broth an Endo plate was streaked and isolations were made on nutrient agar from selected colonies.

The cultures employed at the beginning of the experiment consisted of 15 strains of *Esch. coli* and 9 of *Aerob. aerogenes.* These cultures had all of the general characteristics of typical *Esch. coli* and *Aerob. aerogenes.*

During the first year these soil cultures were tested at about bi-monthly intervals. At the end of the year, all cultures were living. The characteristics of each re-isolated culture were determined; there were few changes. In a few cases a change occurred in the production of indol. This point will be discussed later.

At the end of the second year no cultures were recovered from 7 of the *Esch. coli* inoculated soil tubes, and from 4 of the *Aerob. aerogenes* inoculated soil tubes. A study of the characteristics of the recovered cultures indicated no change from the original.

Three years after the beginning of the experiment 6 of the *Esch. coli* and 3 of the *Aerob. aerogenes* cultures were alive. These isolations had the same characteristics as the original cultures.

At the end of three years and seven months, there remained alive 6 strains of *Esch. coli* and 2 of *Aerob. aerogenes.* A study of these isolations revealed no departure from the original characteristics.

DISCUSSION

Under the described environmental conditions, there was a decided constancy of strain characteristics. In no case was there any indication of reversion. Objections may be raised that this work proves nothing because there were not enough cultures employed. The validity of such a claim is recognized, and these experiments do not prove that bacterial constancy under the described conditions is absolute. Nevertheless, it can be assumed that these results indicate a possibility of what can be expected if a larger number of cultures are used.

The production of indol in a few instances showed some variation; e.g., although an isolation of a specific culture at a certain time might be indol-positive, the next isolation of this strain would be indol-negative. On the basis of these few results, there is a possibility that indol production by any given strain may be a variable characteristic.

Revis (1909) reported a change in the physiological properties of *Esch. coli* when kept in sterile soil. In the present experiments there was no change in the action of any strain on lactose, sucrose, glucose, xylose, dulcitol, mannitol, salicin, citrate, uric acid and gelatin.

The fact that some of these cultures were viable after almost four years is an indication of the protective influence of the soil on these two species, even if this medium is thoroughly air-dried. These findings regarding viability substantiate the report of Young and Greenfield (1923) and also the more recent findings of Tonney and Noble (1931).

SUMMARY

Cultures of *Esch. coli* and *Aerob. aerogenes*, held for long periods of time in sterilized garden soil, did not show any marked change in their physiological, morphological and cultural characteristics.

Eight of 24 strains employed could be isolated from the soil cultures after three years and seven months in this medium.

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ACID PRODUCTION AND TOLERANCE OF LACTOBACILLI FROM DENTAL CARIES AND OTHER SOURCES AS MEASURED BY THE GLASS ELECTRODE[1]

THEODOR ROSEBURY

Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York

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The uniform occurrence in dental caries of aciduric bacteria belonging to the genus Lactobacillus, first clearly demonstrated by Kligler and Gies (1915), and since repeatedly confirmed, and the circumstance that the acidogenic properties of this genus fit in well with the commonly accepted Miller (1890) theory of caries etiology, make it probable that these organisms are involved in the production of carious lesions in teeth. A precise determination of their acidific relations has, however, been wanting; the literature on the subject deals chiefly with rough pH determinations in routine cultures, and is somewhat conflicting. Results reported by previous investigators are given in table 1. The range of variation in these results—pH 2.2 to 4.8—is considerable; and although values obtained colorimetrically are in fair agreement, the discrepancy between them as a group and the values obtained by McIntosh et al. (1922) with the hydrogen electrode is particularly noteworthy. To some extent these variations are probably a reflection of differences in experimental conditions, but differences in the method of measuring pH are probably responsible in greater measure. This investigation aimed to resolve these difficulties by the application of a precise method under suitably standardized conditions, with the object of determining the limiting levels, in terms of pH, of acid production and acid tolerance for lactobacilli.

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[1] **Conducted with the aid of a grant from the Commonwealth Fund for research in dental caries.**

METHODS

a. Measurement of pH

Determinations of pH were made with a glass electrode, employing a vacuum tube potentiometer assembly similar to that recently described by F. Rosebury (1932).[2] For the purpose of these tests electrometric pH measurement appeared desirable, both because of their added precision as compared with colorimetric methods, and because the latter, employing bromphenol blue in the desired range, are subject in this instance to a serious subjective error, owing to the fact that the indicator color is often badly masked in bouillon. A series of preliminary tests with the quinhydrone electrode, checked directly against the glass electrode, showed pH differences between the two instruments ranging from 0 to 0.3 pH above pH 5.0, and considerably larger and variable errors at lower pH levels. The potential of the quinhydrone electrode was subject in the media tested to considerable drifts, generally at pH levels below 7, approaching the corresponding glass electrode value logarithmically, but often failing to reach it within ten to twenty minutes. Because of the variability of

TABLE 1

Final pH of Lactobacillus cultures

INVESTIGATOR	YEAR	SOURCE OF STRAINS	METHOD USED	RESULTS pH
McIntosh et al.	1922	Extracted teeth	Hydrogen electrode	2.2–3.4
Rodriguez	1922	Extracted teeth	Not stated	2.9–3.9
Kulp and Rettger	1924	Intestine, milk	Colorimetric	3.6–4.0
Bunting et al.	1926	Mouth	Colorimetric	4.0–4.2
Morishita	1928	Mouth	Not stated	3.0–3.8
		Intestine	Not stated	3.8–4.4
Rosebury et al.	1929	Mouth, intestine	Colorimetric	3.5–4.8
Hadley et al.	1930	Mouth, intestine	Colorimetric	3.8–4.1
Beckwith	1931	Mouth	"Electrometric"	3.3–3.7
		Intestine	"Electrometric"	3.7–4.3

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[2] I wish to thank Professor H. T. Clarke for permission to use the instrument in the Department of Biological Chemistry at this institution for these tests, and for lending the services of Mr. F. Rosebury, who has since constructed a similar instrument for our own use.

these errors, calibration of the quinhydrone electrode appeared impossible. The glass electrode, on the other hand, seems the instrument of choice for precise bacteriological work, since it appears not to be subject to the errors of other methods (see MacInnes and Dole, 1929, and Dole, 1931); although at present no ultimate standard, directly referable to measurements in culture media, is available.

Potentials were measured by means of a Leeds and Northrup Type K potentiometer. The glass electrode assembly was standardized by means of buffers checked against the hydrogen electrode,[3] and all determinations were made by direct interpolation between standards of known pH, the pH being calculated from the potential value by means of the equation given by DuBois (1930). The average error of these measurements was approximately 0.03 pH.

b. Bacterial strains employed

Nineteen strains of lactobacilli obtained from various sources were studied, as follows:

Nos. 2, 3, 8, 11, 13, 14, 17E, 19, 63, strains from human mouths with dental caries, isolated prior to December 15, 1930.
Nos. 22, 24, strains from human mouths with dental caries, isolated January 12, 1931.
Nos. I, II, IV, V, VII, XI, strains from normal rat mouths, isolated January 5, 1931.
Nos. Ac42, 1482E, intestinal strains (*L. acidophilus*) obtained through the courtesy of Dr. N. Kopeloff, of the New York State Psychiatric Institute.

Strains of *Staphylococcus aureus* and *Bacterium coli* used for comparative purposes were obtained from the stock culture collection of this department.

The results reported herein were obtained between April and July, 1931, after most of the strains had attained a measure of stability in their growth characteristics. Such stability is, however, apparently attained by some strains only after a much longer period, as certain of the results reported indicate.

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[3] I am indebted to Professor C. F. Failey of the Department of Biological Chemistry for these hydrogen electrode determinations.

The colony form exhibited by the strains of lactobacilli was in general as follows: "Smooth" strains: 2, 3, 13, 17E, 19, II, V, Ac42; "Rough" strains: 11, 22, I, XI; highly variable or intermediate strains: 8, 14, 63, 24, IV, VII, 1482E. Most of the strains showed occasional variant colony forms on agar, but since this feature was studied only incidentally, no attempt was made at pure line dissociation. The strains, after primary isolation from single colonies on 1 per cent glucose infusion agar, were carried in 2 per cent glucose stock veal broth, transferred by pipette from broth to broth with only occasional platings to insure purity.

c. *Method for the determination of acid production limits*

The stock veal broth, used for routine cultures and as a basis for all test media, was prepared as follows: 60 grams of Bacto-veal were added to a liter of boiling water, boiled thirty minutes, filtered once through paper, and the volume restored by washing through the filter. One per cent of Bacto-peptone was then added. For stock purposes 2 per cent of Bacto-dextrose and 0.5 per cent of NaCl were added, and the broth was titrated to a pH of about 7, and autoclaved.

As a test medium for the determination of acid-production limits, the broth, before adding the sugar and salt, was diluted, adjusted to the desired pH levels by electrometric titration, and the final medium then made up to a 1:6 dilution of the primary constituents, but with the original sugar concentration. The final pH of a given culture of a lactobacillus, other conditions being held constant, will depend on two factors—the extent of growth of the culture, as influenced by the nutritive properties of the medium, and the buffer strength of the medium. In media which do not contain buffer substances specifically added as such, since the buffer strength is bound up with the nutritive properties of the amino acids of the medium, both factors will vary with the extent of dilution of the broth, within limits. By preliminary experiment it was determined, nevertheless, that in a medium diluted 1:6, but with the sugar concentration made up to 2 per cent, the balance of these factors is such as to result in terminal pH values appreciably lower than can be obtained in a stock broth even though the dilute medium is less favorable to growth. The final pH of a culture of lactobacilli is also influenced slightly by the initial pH of the sterile broth. Accordingly a graded series of broths was prepared, at pH levels ranging from 2.8 to 6.5, on the basis of preliminary electrometric titration. The incompletely diluted medium was adjusted in bulk to pH 2.5 with HCl, and a titration curve plotted by means of a series of tubes containing graded amounts of NaOH, made up to final dilution, covered with paraffin oil, and autoclaved for ten minutes at 15 pounds pressure. The media were then prepared, by suitable interpolation on the titration curve, at pH 2, 8, 4.0, 5.4, 6.0 and 6.5, tubes in 10-cc. amounts, covered with paraffin oil, and autoclaved as before. Paraffin oil was used to prevent evaporation of the broth; it also produced some degree of anaerobiosis, which augmented growth slightly and further decreased the pH levels obtained. The pH of this broth was found to remain constant over a period of two months at room temperature.

Cultures, before being seeded into these media, were stepped up in growth by several transfers through stock broth with pipette (0.5 cc. inocula) at twenty-four-hour intervals. Twenty-four-hour cultures were then centrifuged, washed, and resuspended in a quantity of 0.5 per cent NaCl equal to approximately half the broth removed. The number of organisms in 1 cc. of the suspension was determined by the use of a Petroff-Hausser counting chamber, and a quantity of suspension containing between 30 million and 35 million organisms seeded into each tube of a series. The amount of added material was made up in each case to 1.0 cc. with 0.5 per cent NaCl. This inoculation was equivalent to an average inoculation of 0.25 cc. of broth culture. The actual amounts of suspension used varied between 0.05 and 0.80 cc. These cultures were incubated for seven days at 37°C., and the pH determined.

d. Method for the determination of acid tolerance limits

For this purpose stock broth was used without dilution, adjusted by titration with HCl, at intervals of 0.25 pH between pH

TABLE 2

Data on acid production and growth strength

STRAIN	DILUTE BROTH TUBE NUMBER									MEAN SIGNIFICANT pH	pH IN STOCK BROTH	COUNTS IN STOCK BROTH		
	1		2		3		4		5			1	2	Average
	Controls pH													
	6 53 6 54 6 55		6 00 6 00 5 97		5 34 5 37		3 97 3 98 4 00 4 02		2 81 2 84					
	Control average													
	6 54		5 99		5 36		3 99		2 83					
	pH	pH change	pH	pH change	pH	pH change	pH	pH change	pH					
												millions per cc.	*millions per cc*	*millions per cc.*
2	3 32	3.22	3 20	2.79	3 24	2.12	3 15	0.84	2 83	3 23	3 68	1,236	1,080	1,158
3	3 53	3.01	3 59	2.40	3 42	1.94	3 10	0.89	2 84	3 41	3 86	670	400	535
8	3 52	3.02	3 36	2.63			3 17	0.82		3 35	3 77		456	456
11	3.36	3.18	3 35	2.64	3 24	2.12	3 11	0.88	2 81	3 27	3 72	464	678	571
13	3 49	3.05	3 38	2.61	3 40	1.96				3 42	3 78	404	303	353
14	3 61	2.93	3 64	2.35	3 69	1.67	3 48	0.51		3 61	4 32	244	212	228
17E	4 27	2.27	4 16	1.83	4 00	1.36	3 77	0.22		4 05	4 15	212	202	207
19	4 01	2.53	3 62	2.37	3 64	1.72	3 47	0.52		3 69	4 26	196	220	208
22	3 54	3.00	3 60	2.39	3 54	1.82	3 26	0.73		3 49	4 32	232	184	208
24	3 70	2.84	3 68	2.31	3 65	1.71	3 54	0.45		3 64	4 10	296	254	275
63	3 46	3.08	3 37	2.62	3 36	1.97	3 13	0.86		3 33	3 83	690	546	618
I	4 48	2.06	4 29	1.70	4 19	1.17	4 03	0.00		4 32	4 10	552	142	347
II	3 76	2.78	3 71	2.28	3 68	1.68	3 35	0.64		3 63	3 95	104	136	120

IV	3.67	**2.87**	3.73	**2.26**	3 58	**1.78**	3.40	**0.59**		3 60	3.92	156	184	170
V	5.00	**1.54**	4.22	**1.77**	4 01	**1.35**	4 01	**0.00**		4 41	4.42	112	80	96
VII			4.12	**1.87**	4.11	**1.25**	4 19	**+0.20**		4 11	4.26		172	172
XI	3.81	**2.73**	3 78	**2.21**	3 72	**1.64**	3 57	**0.42**		3.72	4 52	78	306	192
Ac42	3.30	**3.24**	3.23	**2.76**	3 22	**2.14**	3 58	**0.41**		3.33	3.65	378	720	549
1482E	3.24	**3.30**	3.26	**2.73**	3 21	**2.15**	3 30	**0.69**	2 80	3.25	3.60	508	824	666
B. coli	4.20	**2.34**	4.13	**1.86**	4 08	**1.28**	4 11	**+0.12**			4.76			
S. aureus	4.18	**2.36**	4.12	**1.87**	4 26	**1.10**	4 10	**+0.11**			4.60			
Averages:														
Group I.....	3.42	**3.12**	3.37	**2.62**	3 33	**2.03**	3 23	**0.77**						
Group II.....	3.76	**2.78**	3.69	**2.30**	3.66	**1.70**	3 47	**0.52**						
Group III....	4.58	**1.96**	4.20	**1.79**	4.08	**1.28**	4.00	**0.00**						

2.3 and 4.8. These were seeded with 0.1 cc. of twenty-four-hour broth cultures, incubated for eighteen hours at 37°C., and all tubes were then plated out by streaking on 1 per cent glucose agar. Viability at the different pH levels was determined by growth on the agar after forty-eight hours' incubation at 37°C.

RESULTS

a. Acid production tests

The pH values obtained in the cultures in dilute broth are given in table 2. At the head of each column are the values obtained at different times on uninoculated control tubes incubated with each batch. The average of these values is taken as the initial pH level of the group. No growth was apparent in any instance at pH 2.8, and since the most strongly acidogenic strains failed to depress the pH below this level, many of the tubes were not tested. The value given in the table under Mean Significant pH is obtained by averaging the values for the given strain which indicate a depression of the initial pH level of the medium. By dividing the strains into three groups on the basis of this value: group I, below 3.5; group II, between 3.5 and 4.0, and group III, above 4.0, and plotting the average pH change for groups I and II against the initial pH of the medium at each level, the linear graphs shown in figure 1 are obtained. The upper dash line represents a uniform final pH of 3.0. Extrapolation of the graphs to the base line (representing zero pH change) provides a means of approximating the group limits of acid production. It appears that the lowest of these limits (that for group I) is a value slightly above pH 3.0. This value is, indeed, not exceeded by any of the individual pH values obtained; the lowest value, obtained in the pH 4.0 broth with strain No. 3, being 3.10.

The values for group III, whether because they are not sufficiently numerous, or because the group is less homogeneous than the others, do not plot as a straight line and are not included in the graph. The difference between this group and *B. coli* and *Staph. aureus* is, in fact, too small to permit differentiation.

In another column of table 2 are given a group of pH values of old cultures in stock broth, obtained a month to three months previously to the other tests. The differences between these values and those in dilute broth clearly show the advantage of the latter in this respect; lack of correlation in a few instances is to be attributed to the strain instability previously mentioned. These values, it may be noted, lie in the same range as results obtained colorimetrically by investigators in the past (table 1).

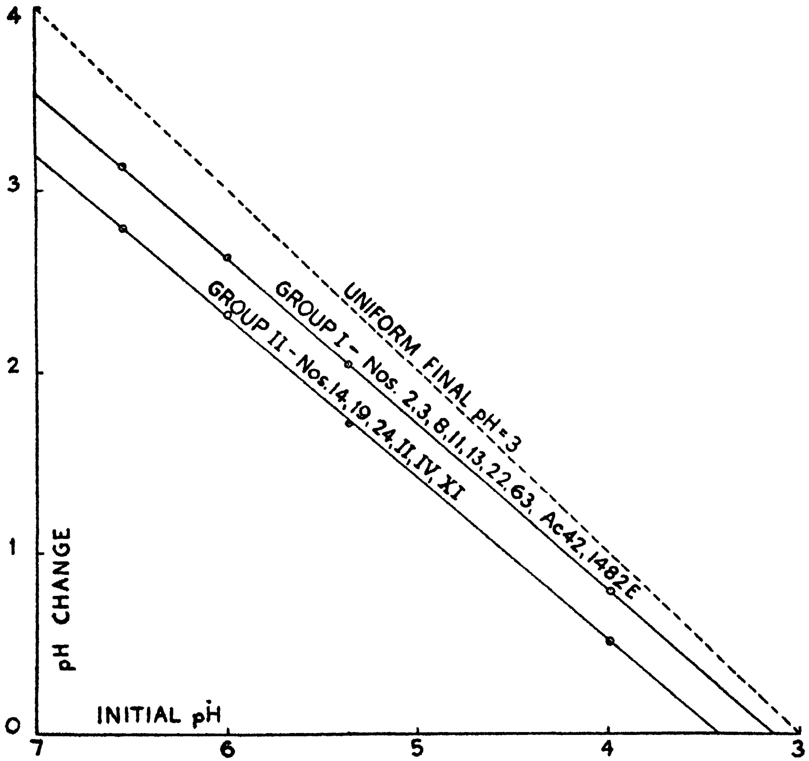

Fig. 1

In figure 2 the mean significant pH is plotted against the growth strength of the strain, the latter being laid out on logarithmic scale. Only two counts for each strain were averaged for this purpose, and no claim to great accuracy can be made; yet it will be seen that the correlation is in general very good. Since both of these characteristics—growth and acid-production—are among the most stable characteristics of the strain, and appear to be independent of the number of organisms inoculated into the medium, this correlation may be taken as indicating the close biological relationship of all of these strains. This point is discussed below.

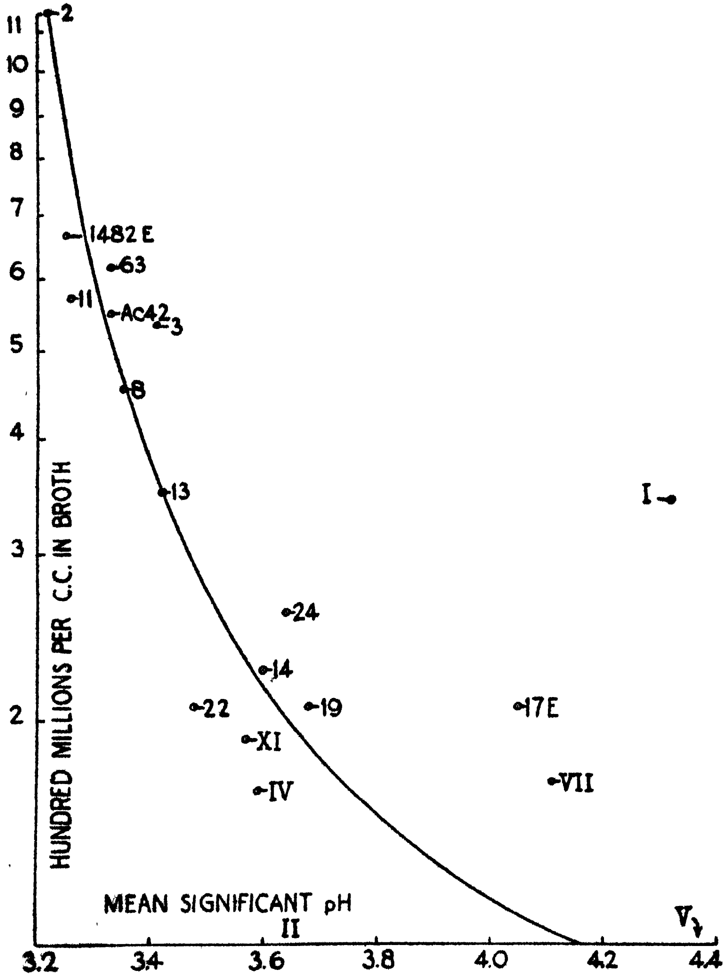

Fig. 2

b. Acid tolerance tests

Six strains, selected from the three groups previously described, were tested in the range pH 2.3 to 4.8. The results are given in table 3. Since no growth occurred in any case in the plates prepared from the tubes of lower pH, some of these are omitted from the table. On the basis of this test a second series was run with all the strains studied, at two pH levels only, pH 3.5 and 4.0, levels which from table 3 appear to have differential significance. In this series, however, all strains except no. I remained viable after eighteen hours at both levels, strain no. I failing to grow out from the pH 3.5 broth. These results are, accordingly, no more than suggestive. As far as they go they indicate that acid tolerance parallels acid production. *B. coli* and *Staph. aureus*, on the other hand, show viability at lower pH levels than either is able to produce in the process of growth. This may be considered as one manifestation of the general resistance of these species.[4]

TABLE 3

Viability (by glucose agar streak) after eighteen hours in HCl-stock broth at pH

STRAIN	4.80	4.48	4.28	4.05	3.83	3.58	3.32	3.04	2.78
2	+	+	+	+	+	+	+	0	0
8	+	+	+	+	+	+	+	0	0
1482E	+	+	+	+	+	+	+	0	0
24	+	+	+	+	+	+	+	0	0
XI	+	+	+	+	0	0	0	0	0
I	+	+	+	+	0	0	0	0	0
B. coli	+	+	+	+	+	+	0	0	0
S. aureus	+	+	+	+	+	0	0	0	0

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[4] Experiments begun since the completion of the work described in this paper indicate that these strain differences in acid tolerance among lactobacilli tend to disappear if a similar number of organisms is inoculated into the test media in each case. It appears that, whereas acid production may be made to approach a true limit, as is here attempted, the level of tolerance may be varied theoretically without limit by varying two factors— the number of organisms inoculated, and the length of time they are exposed to the acid medium. The values for acid tolerance herein given have, accordingly, no absolute significance, but are valid only for the conditions stated.

No correlation between colony form and any of the properties of lactobacilli studied can be made out from the data available.

DISCUSSION

If we may accept the values herein reported as accurate within the experimental error of the method, the results reported by McIntosh et al. (1922), given in table 1, are invalidated, as are likewise the lower values reported by Rodriguez (1922) and Morishita (1928). The correspondence of these glass electrode values with colorimetric values previously reported is, on the other hand, an indication of the validity of both methods.

The data presented in this report appear to throw some additional light on the interesting question of classification of lactobacilli. The writer, in a previous communication (1929), failed to confirm the observation of Morishita (1928) that differences between the dental and the intestinal groups of lactobacilli are sufficiently marked to warrant differential classification. Our observations have since been confirmed by Howitt (1930) and by Hadley et al. (1930); but, because of the variability in morphology, serology, and biochemical properties of individual strains within the group, the question cannot yet be regarded as settled. Additional evidence, however, which points to a similarity of lactobacilli from the three sources herein employed is afforded by the present investigation on two grounds. First, the correlation between growth strength and acid production represented in figure 2 suggests a fundamental similarity in metabolic activity; secondly, the two intestinal strains studied fall well within the strongest of the arbitrarily designated acid-production groups,—group I, which contains the majority of the dental strains—a finding at variance with the differences reported by Morishita (1928), and, recently, by Beckwith (1931). On the other hand, differences which might be ascribed to habituation, and which might tend to disappear on prolonged cultivation under uniform conditions, such as the criterion, for *L. acidophilus*, of human intestinal survival, suggested by Upton and Kopeloff (1931), may doubtlessly be useful for practical purposes, and need not involve the fundamental matter of taxonomy. From this viewpoint, the grouping herein devised, on the basis of acid production, whether it be stable or the expression of a mere transient condition of a bacterial strain, may nevertheless be of practical value in the study of the etiology of dental caries. In view of the hypothesis that dental caries is a result of the acid concentration produced by lactobacilli at the tooth surface, it seems suggestive that of the strains studied in this investigation, those derived from mouths in which caries was present are aligned, with one exception, with the stronger acid producers, while those derived from mouths (of rats) in which no caries occurred[5] are aligned with the weaker. The data on acid tolerance given here contain a suggestion of similar alignment in this respect. Further studies are now in progress on the respective acid tolerance of lactobacilli, from mouths in which caries is active, and from mouths having a history of complete absence of caries, both at time of isolation and subsequently; and of their respective acid production after acclimatization to artificial cultivation.

SUMMARY

Nineteen strains of lactobacilli, including human dental and intestinal forms and strains from normal rat mouths, and one strain each of *B. coli* and *Staph. aureus*, were tested for acid production limits in a dilute veal broth titrated to pH levels between 2.8 and 6.5, and for acid tolerance limits in a stock veal broth titrated to pH levels between 2.3 and 4.8. All pH determinations were made by means of a glass electrode, employing a vacuum tube potentiometer. The results indicate that strain differences characteristic of lactobacilli in other relations are also apparent in this respect; the most strongly acidogenic strains, including members from the dental and intestinal groups, are able to attain to a pH slightly above 3 as the initial pH of the medium is progressively reduced; the weakest strains, including chiefly rat forms, do not reduce the pH of the medium below 4, and are in

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[5] The rats from which these strains were obtained were the subjects of another investigation under way at this time in this laboratory. The jaws and teeth of these animals have since been sectioned and examined microscopically. No dental caries was present.

this respect similar to *B. coli* and *Staph. aureus*. A correlation between growth strength and acid production is indicated. Tests for acid tolerance limits, thus far incomplete, indicate that this property parallels acid production for a given strain.

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SOME OBSERVATIONS ON CHITIN-DESTROYING BACTERIA[1]

DELIA E. JOHNSON[2]

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While attempting to prepare chitin from the exoskeleton of some hard shell crabs (*Cancer magister*), procured at a local market, the writer found some specimens of this shell fish to have an extremely foul odor, although they had been packed in ice at the market. The odor evidently emanated only from the exoskeleton, for the flesh, when removed from the exoskeleton, seemed to be free from any off-odor. Since the exoskeleton of the hard shell crab is composed largely of chitin and calcium, this odor suggested the presence and activity of chitin bacteria. Mounts made from the scrapings from the exterior of the exoskeleton and examined under the microscope showed rod-shaped and coccus-like bacteria. Their appearance corresponded somewhat with that of *Bacillus chitinovorus* as described by Benecke (1905) of Germany. Folpmers (1921) corroborated Benecke's findings and also isolated a strain which differed from Benecke's bacterium in its action on gelatin. The writer (1931) described a Myxococcus antibiotic to young cultures of the corn smut fungus, *Ustilago zeae* (Beckm.) Ung., and the oat smut fungi, *Ustilago levis* (K. and S.) Mag., and *Ustilago avenae* (Pers.) Jens. which also broke down chitin. It was therefore decided to determine if any of these cultures might be responsible for the odor of putrefaction of the hard shell crab.

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[2] The writer wishes to express her appreciation to Dr. E. C. Stakman, of the Division of Plant Pathology and Botany, and to Dr. A. T. Henrici and Dr. C. E. Skinner, of the Division of Bacteriology, for their interest and helpful suggestions. She also is indebted to Dr. Samuel Eddy, of the Division of Zoology, for the identification of the hard shell crab.

Some of the exoskeleton of the hard shell crab was purified by Benecke's method, and pieces of it were added to the type of agar medium which he used. This medium was then inoculated with small pieces of the odorous exoskeleton and left to incubate at room temperature. Sterile water was added to keep the chitin moist and to allow both aerobic and partially anerobic conditions. Commercially prepared chitin (from Eastman Kodak Company) was used in the same way. Checks of both types of cultures were handled in the same manner as the chitin cultures. In a few days the solution above the agar became cloudy in some of the inoculated flasks, and a microscopic examination showed many types of bacteria. After a few weeks it became evident that the commercial chitin and the purified exoskeleton were being utilized by the bacteria, both being gradually broken down. By repeated transfers to fresh chitin media, a more uniform type of culture was obtained.

It is evident that the decomposition products of the chitin can support growth of various types of bacteria, and this accounts for the presence of the non-chitin destroying bacteria in the cultures. Single colony cultures were obtained by plating on Kofler's (1913) sucrose medium, and inoculations were again made into chitin media. In this manner many single colony cultures were obtained which broke down both the commercial chitin and the purified exoskeleton of hard shell crab. None of the cultures grew upon the exoskeleton of cray fish and other fresh water Crustacea used.

To determine whether the same types of bacteria could be found on plants containing chitinous material, inoculations were made into chitin media from decaying tissue of the fruiting bodies of the honey cap mushroom, *Armillaria mellea*, and other Basidiomycetes. A number of cultures of a slightly different type were obtained from these sources.

After a period of incubation, the chitin in some of the flasks became covered with small salmon-colored to pink fruiting bodies, consisting of round spore-like bodies. Cultures from these developed into rod-shaped forms. This type of development corresponds to that of Myxococcus, a genus of the Myxobacteriaceae. All the non-fruiting cultures resembled the vegetative form of Myxococcus in general, and some of them produced round spore-like bodies in old cultures, but no fruiting bodies could be found upon any of the media used. There is some variation in the ability of Myxococcus cultures to fruit on the same types of media, so possibly, if the right medium could be supplied, the non-fruiting cultures would fruit.

Since so much work has been done on the morphology of the myxobacteria, a description of the order is not necessary. The physiology of the group, however, has not been studied adequately. Until such a study is made it would be useless to try to identify the chitin bacteria mentioned in this article. They are, therefore, referred to by their laboratory number, and described only very briefly, in table 1.

It might be of interest to mention that, although *Myxococcus rubescens* is one of the most common of the pink-fruiting types, none of the cultures mentioned here seem to belong to this species, for several investigators have observed that it liquefies agar, while none of the chitin destroyers studied by the writer had this effect. One pink-fruiting type was found which liquefied agar, but it did not break down chitin.

Culture 104 is interesting because it affects only certain pieces of the commercial chitin which it turns black, and has no effect on the exoskeleton of the hard shell crab. Commercial chitin probably contains chitin from many sources, and possibly these are not all equally available to this bacterium.

In the accompanying plates, figure 1 shows the check, the approximate amount of commercial chitin broken down in about two months by a straight needle inoculation from this chitin culture. Figure 2 shows the chitin softened by this organism until it could be smeared on a slide and stained. Figure 3 shows a check, the approximate amount of purified exoskeleton which was crumbled down to the form shown in figure 4. The bacterial action is continued on the exoskeleton until only the calcium remains.

Many of these cultures grow upon the flesh of the crab. When the flesh is not removed entirely from the exoskeleton, the latter seems to be broken down much more rapidly. This is probably comparable to what takes place in nature. All of the cultures lose their chitin-destroying property when cultivated for some time upon media containing no chitin. Not all species of Myxococcus break down chitin, at least ten cultures of different types having been obtained from the same sources which had no effect on chitin whatever.

TABLE 1

LABORATORY NUMBER OF CULTURES	SOURCE	SPORULATION*	FRUITING BODY COLOR	TYPE OF BACTERIUM	TYPE OF GROWTH ON SPECIAL MEDIUM	
					On chitin	On Kofler's
80	Hard shell crab	+	Pink to salmon	Myxococcus	Slimy; cream colored	Slimy; medium darkened
90	*Armillaria mellea*	+	Pink	Myxococcus	Slimy; lemon yellow	Scant; white
95	*Armillaria mellea*	+	Pink in areas of yellow slime	Myxococcus	Yellowish	Lemon yellow
97	Hard shell crab	+	Purple	Myxococcus	Purple	No growth
71	Hard shell crab	+	None found	?	White-yellowish	No slime; cream colored
82	Hard shell crab	+	None found	?	Dark colored	Scant; greenish
98	*Armillaria mellea*	−	None found	?	Dirty yellow	Scant; dirty yellow
104	Hard shell crab	−	None found	?	Black; only certain pieces affected	Dirty yellow
92	Hard shell crab	This bacterium could not be cultured on other than chitin media. It could not be separated in chitin cultures from a bacterium which did not affect chitin.				

* + = spores observed; − = none found.

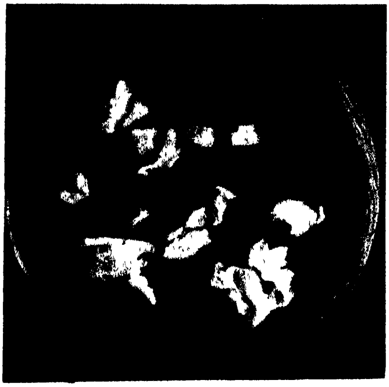

FIG. 1 Check, showing the commercial chitin remaining unchanged upon the agar This is the approximate amount of chitin broken down by culture 92 in two months from a straight-needle inoculation from a water suspension of the bacteria Dried to facilitate photographing (×⅔ original size.)

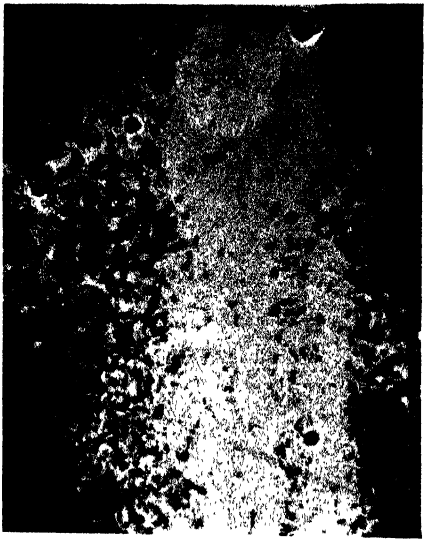

FIG 2 A stained smear of the softened chitin.

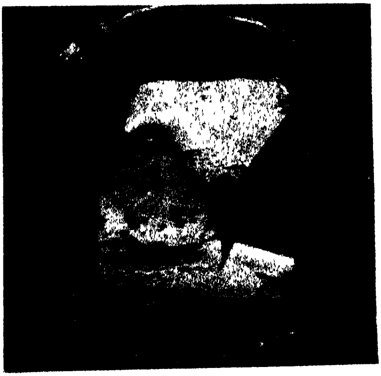

FIG 3 Check showing the approximate amount of purified exoskeleton of hard shell crab broken down by culture 92 in about two months (×⅔ original size)

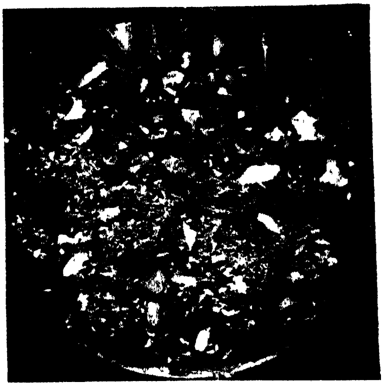

FIG 4 Fragments of exoskeleton showing the result of the bacterial action. (Both 3 and 4 were also dried before photographing)

SUMMARY

A discussion is given of several kinds of chitin-destroying bacteria obtained from malodorous hard shell crabs and from the decomposing fruiting bodies of several Basidiomycetes.

These bacteria also grew upon the flesh of the hard shell crab, and destroyed chitin much more rapidly when the flesh was not entirely removed from the exoskeleton. It is therefore possible that they may be somewhat important in destroying chitinous material in nature.

One of the organisms studied could be cultivated only upon chitin media, and all the others lost their chitin-destroying property when cultivated for several weeks upon agar media.

Some of the organisms produced fruiting structures typical of Myxococcus, and the other cultures had the characteristics of the vegetative stage of Myxobacteria.

Not all of the Myxococcus types studied destroyed chitin.

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EFFECTS OF PANCREATIC ENZYMES ON THE TUBERCLE BACILLUS

W. N. BERG

Berg Biological Laboratory, New York City

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The literature contains confusing statements in regard to the effects of digestive enzymes on living or dead tubercle bacilli. To release tubercle bacilli imbedded in sputum, Spengler (1903) digested the sputum with pancreatin—a method evidently based upon the assumption that living bacilli are not killed by this treatment.

Statements or implications that the live tubercle bacillus resists digestion may be found in the publications of Jobling and Peterson (1913), Baldwin, Petroff and Gardner (1927), and Day and Gibbs (1930).

Loeffler (1913) mixed 5 mgm. of live tubercle bacilli with 20 mgm. of trypsin in a volume of 2 or 3 cc. After three days' contact, the bacilli were no longer infectious for guinea pigs nor were growths obtained in subculture. Sometimes one day's contact killed all the bacilli. Loeffler (1913) did not record any results on the extent to which the bacilli were digested. The staining reactions of the enzyme-killed bacilli were the same as those of the controls.

Control tests indicated that the killing effect was due to the enzyme and not to other constituents of the suspension fluids. "Carnevorin," an enzyme preparation from Drosera, the insect-eating plant, was used to study its effects on a number of micro-organisms suspended in it. The shortest killing times were: anthrax bacillus, ten minutes; bacillus of swine erysipelas, twenty minutes; bacillus of mouse typhoid, sixteen hours; tubercle bacillus, one to three days. Judging from this publication alone, Loeffler (1913) concluded that live tubercle bacilli were digested by the enzymes used, thereby killing them. Search of the literature showed that Loeffler's subsequent publications were on military surgery. A continuation or revision of the foregoing work was not found.

Robinovitch (1926) found that live tubercle bacilli were rendered non-acid fast in one-half minute when mixed with concentrated sterile filtered glycerol-pancreas extract. Guinea pigs injected two to four hours later with this mixture, escaped infection.

Day and Gibbs (1930) suspended live tubercle bacilli in sterile pancreatic juice obtained from dogs. They concluded ". . . . while *B. tuberculosis* was killed by sterile pancreatic juice, no evidence of dissolution of cells or loss of acid-fast property was observed . . . . " after forty-eight hours' contact. Their paper did not mention any direct proof that the bacilli were not digested. Apparently, this was inferred from the retention of acid-fastness and microscopic appearance.

DISCUSSION

The guinea pig and subculture tests as used in the three foregoing investigations probably gave imperfect measures of the shortest killing time. The procedures measured the safe time interval within which all the tubercle bacilli were killed. Even if 90 per cent of a loopful of live tubercle bacilli suspended in glycerol-pancreas extract were killed in an hour, the remaining millions of bacilli would still prove infectious. Perhaps sharper results would be obtained if the enzyme were mixed with filtered suspensions containing single bacilli only, such as one prepares by Jenning's (1926) method for counting the bacilli in the Petroff-Hausser counting chamber.

The present work has dealt with two problems: (1) the extent to which tubercle bacilli are digested by pancreatic enzymes acting together and (2) the possible use of such split tubercle bacilli to protect laboratory animals against virulent strains. Only the first part is reported here. With regard to the second part: encouraging results were obtained in 1930 and 1931 with two series of guinea pigs. Attempts are now being made to protect rabbits. The results, it is hoped, will appear in another paper.

CULTURES

Twelve human and 4 bovine strains of tubercle bacilli were studied. The writer gladly acknowledges his indebtedness for these cultures to Dr. Theobald Smith, Rockefeller Institute, Princeton, N. J., Dr. H. J. Corper, National Jewish Hospital, Denver, Colo., Dr. Marion Dorset, Bureau of Animal Industry, Washington, D. C., and Dr. S. A. Petroff, Trudeau Sanitarium, Trudeau, N. Y. Most of the strains had recently been isolated. These were grown on Long's (1926) synthetic medium. Its composition is: water, 1,000 cc.; asparagin, 5 grams; ammonium citrate, 5 grams; acid potassium phosphate, 3 grams, anhydrous sodium carbonate, 3 grams; sodium chloride, 2 grams; anhydrous magnesium sulphate, 1 gram; ferric citrate 0.062 gram; glycerol, 50 grams (40 cc.), and agar, 20 grams. Different samples of "pure" glycerol may contain varying amounts of acid. To facilitate the routine preparation of the medium, the acid was neutralized by adding about 30 grams powdered calcium carbonate to 1 kgm. glycerol and filtering. The finished medium had a pH close to 7.6.

ENZYME PREPARATIONS

Four commercial preparations in powder form, were used. Each of these probably contained a varying mixture of several enzymes. Numerous blank tests were made to determine the necessary corrections in the digestion data.

Holadin, a pancreatic preparation, is made by Fairchild Brothers and Foster, New York City. It contained amylase, lipase, trypsin and esterase. Other enzymes were not tested for.

Trypsin, made by Wilson and Co., Chicago, Ill.

Pancreatin and Taka-diastase, both made by Parke Davis and Company, New York City.

An advantage in beginning the work with pancreatic preparations was that the enzymes they contain are active in nearly neutral solutions. Complication due to subjecting tubercle bacilli to strong acids or bases is thus eliminated.

DIGESTIBLE CARBOHYDRATE—SERIES OF JUNE, 1928, TO MAY, 1929

Method

Tubercle bacilli of a single strain, nearly dry, were transferred from one or more agar slants or "French square" bottles, into a weighed, sterile 50-cc. Erlenmeyer flask. Two grams moist weight of bacilli in one flask was convenient. The bacilli were then suspended in Ringer solution saturated with chloroform so as to contain 50 to 100 mgm. tubercle bacilli per cubic centimeter (see table 1). Portions of the suspension were pipetted into two or more small flasks. To one flask a weighed amount of pancreatic enzyme powder was added. Control tests were made on the synthetic medium with and without added enzyme. The medium contained negligible traces of reducing substances which were omitted from the calculations summarized in table 1. To each flask, 0.5 cc. chloroform and 0.2 cc. toluol were added. The flasks

TABLE 1

Digestion expressed in milligrams glucose increase over total corrections, per 1 cc. tubercle bacillus suspension

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Strain	H37	Sputum 63	Sputum B	Squibb III	Squibb III	Smith VI	Smith VI
Age of tubercle bacillus culture, weeks	5	5	9	12	12	12	12
Weight of fresh tubercle bacilli in 1 cc suspension, mgm.	60	60	100	46	46	57	57
Weight of enzyme powder added to 1 cc suspension, mgm	1 Holadin	1 Holadin	1 Pancreatin	0.1 Holadin	0.1 Taka-diastase	0.1 Holadin	0.1 Taka-diastase
Digestion period	Milligrams glucose increase over corrections						
days							
1	0	0					
7	0.18	0.056					
9			0.03				
15	0.18	0.08		0.039	−0.007	0.025	0
16			0.09				
22	0.02	0.02					
23			0.05				
31				0.06	0.024	0.044	0

were closed with rubber stoppers and kept in the incubator at 37°C.

At intervals of one or more days, small portions of the bacterial and control suspensions were removed and filtered. Copper reduction in the filtrates were determined by Folin's (1926) urine dextrose method and later by Folin's (1926) blood dextrose method. After a number of variations had been tried the following methods were most frequently used. Deviations from Folin's directions are indicated.

Into a test tube, pipet a 1-cc. portion of tubercle bacillus or control suspension. Omit addition of oxalic acid. Add 3 cc. water, 0.5 gram Lloyd's reagent, shake. Omit filtration. Add 0.66 gram permutit, shake. After four minutes centrifuge or filter through a 6-cm. filter paper in a 2.5-cm. funnel. Funnels and papers must be small to avoid mechanical losses of filtrates.

Instead of the foregoing use of Lloyd's reagent and permutit, other filtrates were preferably obtained as follows: Into a test tube, pipet 1 cc. tubercle bacillus or control suspension. Add 2 cc. water, 1 drop or 0.04 cc., 10 per cent sodium tungstate solution and 1 drop or 0.04 cc. 2N/3 sulphuric acid. Usually, the flocculation was good and a perfectly clear filtrate was obtained. Occasionally a second drop of acid was necessary. It is essential that the filtrate be clear, and give no further precipitate when tested by adding a drop of tungstate and acid solutions. Two 1-cc. portions of filtrate are easily obtained from one precipitation and filtration.

Without any delay, 1-cc. portions of filtrate were pipetted into 150 by 15 mm. test tubes. Into 8 similar test tubes, standard glucose solution was pipetted so that they contained 0.003, 0.005, 0.007, 0.01, 0.02, 0.04, 0.06 and 0.09 mgm. in 1 cc. water. To all the tubes, 1 cc. copper reagent was added, followed by heating for ten minutes in a boiling water bath. After the heating, all tubes were quickly cooled in cold water, and 1 cc. molybdate solution was added to each. The total volume in each tube was 3 cc. The colors in the tubes containing tubercle bacillus filtrates were compared with those in the standards. Fine readings or frequent dilutions were not necessary. Occasionally the color in the 0.003 mgm. tube was not noticeable. This tube served to indicate the amount of loss due to copper re-oxidation. The results were recorded as glucose in 1 cc. tubercle bacillus (or control) filtrate, without assumptions regarding the nature of the reducing substances.

Results

Sputum H, a human strain recently isolated by Dr. H. J. Corper at Denver, was first to be studied. Ten digestion experiments involving about 100 glucose determinations, were made using several consecutive transplants on Long's synthetic agar medium. Seven experiments were made on Gluckson, another human strain from Denver. Thirteen experiments were made on three other human strains, H37, sputum B and sputum 63 and on 2 bovine strains, Squibb III and Smith VI. Out of 30 experiments, seven have been selected and summarized in table 1.

Corrections for reducing substances in the enzyme preparations are not shown in table 1. In chloroform Ringer suspension, all the enzyme preparations showed increasing amounts of reducing substances as the age of the suspension increased. For 1 mgm. Holadin the glucose figures were: at one day, 0.001 mgm.; one week, 0.004 mgm.; two weeks, 0.006 mgm.; one month, 0.007 mgm. For 1 mgm. Taka-diastase the glucose figures were: at one day, 0.23 mgm.; at two weeks, 0.33 mgm.; at one month, 0.50 mgm. Such large corrections obscure other data and probably account for the negative figure for Squibb III after fifteen days' digestion. See table 1, column 6.

Corrections for reducing substances in control tubercle bacillus suspensions to which no enzyme had been added are likewise omitted from table 1. The glucose content of these suspensions generally diminished with age. This is probably the arithmetical result of two simultaneous processes: (1) slow digestion of carbohydrate by autolytic enzymes, and (2) a slightly more rapid disappearance of reducing substances through causes unknown at present.

The data for sputum 63 (column 3) may be read as follows: A five weeks' growth of sputum 63 was suspended in chloroform

Ringer solution so that 1 cc. of suspension contained 60 mgm. of nearly dry bacilli. The suspension was divided into two portions. To one portion, 1 mgm. Holadin per cubic centimeter of suspension was added; no enzyme was added to the other portion. After adding chloroform, 0.5 cc., and toluol, 0.2 cc., to each flask, both were incubated at 37°C. along with a suspension of Holadin which contained 20 mgm. in 1 cc. chloroform-Ringer solution. Toluol, which seemed to evaporate through the rubber stoppers was replaced when containers were opened.

The next day, 1-cc. portions were removed, precipitated with sodium tungstate and sulphuric acid and filtered. Glucose figures in the filtrates were, for the suspension plus Holadin, 0.001 mgm.; for the control suspension without Holadin, 0.00 mgm.; for 1 mgm. Holadin, 0.001 mgm. The corrected figure, 0.0 mgm., appears in table 1. It indicates that after sixteen hours' contact, carbohydrate digestion was not detected. The corresponding figures obtained after seven days are 0.066 mgm. from which were deducted 0.007 mgm. in the control suspension and 0.004 mgm. in 1 mgm. Holadin. The corrected figure, 0.056 mgm. glucose per 1 cc. tubercle bacillus suspension appears in table 1. For fifteen days' digestion the figures were 0.09, 0.00 and 0.006 mgm., giving a corrected figure of 0.08 mgm. The figures probably understate the extent of digestion because they represent the difference between glucose (or its reduction equivalent) formed by digestion and glucose removed by oxidative or other chemical reactions in this very complex mixture. Invariably, the glucose figures became zero or nearly zero.

The results with Smith VI show the advisability of using more than one enzyme preparation. The results with Taka-diastase indicate no digestion, perhaps because of the large blanks for reducing substances in Taka-diastase. The results with Holadin give proof to the contrary.

The detection of carbohydrate digestion due to autolytic enzymes and to as little as 0.1 mgm. added pancreatic enzymes to 50 mgm. tubercle bacilli gives little support to the view that the tubercle bacillus is not digestible. Calculated as glucose, the digestible carbohydrate in 1 gram tubercle bacillus varied up to

3 mgm. depending upon the digestion period and other factors. Which polysaccharide was digested cannot be stated. A possibility is the polysaccharide recently isolated by Dorset and Henley (1930) from tuberculins prepared from sugar-free media.

DIGESTIBLE FAT—SERIES OF MAY, 1930, TO JUNE, 1931

The frequent modifications of copper reduction methods made it desirable to check the foregoing conclusions by another method having different errors. If the fat in the tubercle bacillus is digestible by pancreatic or other enzymes, there should be an increase in acidity which might be detected and measured.

Method

One-gram portions of mixed strains of tubercle bacilli were suspended in 9-cc. chloroform Ringer solution in sterile graduated 15-cc. centrifuge tubes. To all tubes including controls, 0.2 cc. chloroform, 0.1 cc. toluol and one drop of 0.02 per cent phenol red indicator solution were added. Weighed amounts of commercial pancreatic enzyme powders were added to the tubercle bacillus suspensions as shown in table 2. The suspensions and their controls were titrated with N/20 sodium hydroxide to a pH close to 7.8 within an hour or two after removing the bacilli from Long's synthetic agar medium. This was done to neutralize the acid in the liquid adhering to the bacilli and in the enzyme preparations. This started the experiment with zero acid in the suspensions. These amounts are recorded in table 2 after digestion period 0, in even numbered columns. The corresponding figure in the odd numbered column represents the same adherent acid plus the acidity of the added enzyme.

In some experiments tubercle bacilli were pressed between filter papers for a few minutes. This removal of adherent liquid lowered the amount of sodium hydroxide necessary for neutralization. In all cases, these amounts of sodium hydroxide were recorded, but not included as sodium hydroxide used to neutralize acid liberated by digestion.

When titrating the suspensions, care was taken to avoid the addition of excess sodium hydroxide. The pancreatic enzymes rapidly lose activity when the pH increases over 8. No attempts were made to match colors with minute exactness. It was only necessary to add sufficient sodium hydroxide to change the phenol red color from acid to pH 7.8 to 8.0 inverting the tubes a few times to insure mixing. All tubes were rubber stoppered and kept in the incubator. When the indicator had changed to acid color, the apparent volume of the tubercle bacillus sediment was recorded and the tubes again titrated to pH 7.8 to 8.0.

TABLE 2

Fat digestion expressed as cubic centimeters N/20 acid liberated by 1 gram mixed strains fresh tubercle bacilli with and without added pancreatic enzymes

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	CORRECTION FOR ENZYME ALONE
Tube number..	YA	YB	AA	AB	D1	D2	E1	E4	F1	F3	
Age of cultures, days	15	15	25	25	26	26	53	53	24	24	
Milligrams enzyme added to 1 gram tubercle bacillus ..	50*	0	50*	0	50†	0	50†	0	50†	0	
Digestion period	Cubic centimeter N/20 sodium hydroxide added										
days											
0	1.1	0.6	1.2	0.9	1.3	0.8	1.3	0.9	1.1	0.7	0.46
1	1.6	0.8	2.5	0.9							
2	2.2	1.5			1.9	0.7	2.9	1.3	1.7	0.9	
3			3.5	1.6	2.9	1.0					0.40
4	3.2	2.3							3.0	1.3	
5					4.4	1.6	4.5	1.9			
6	4.3	2.8	4.5	2.2			5.4	2.6	4.1	1.9	
8			5.0	3.4							
9					5.0	2.9					
10									5.4	2.4	
12	5.4	3.0	6.0								
18	6.4	3.8									0.45
24			7.2	3.9							
28									7.8?	3.2	
36	7.0	4.3									0.56
40					6.9						
52										3.7	
73								4.4			

* 25 mgm. Holadin plus 25 mgm. trypsin.

† Trypsin.

The liberation of acid was rapid at the beginning of the experiments. In a few instances the tubercle bacillus suspensions became acid in fifteen minutes after the preliminary neutralization. Does this mean that fat digestion was manifested in fifteen minutes? It might also mean that fatty acid materials on the outside of the bacilli had slowly gone into solution. The liberation of acid slowed down, until after a month's incubation, with several intervening titrations, fat digestion practically came to an end.

In table 2, 5 experiments out of more than 30 are summarized. Figures in the odd numbered columns show the amount of N/20 acid liberated by 1 gram tubercle bacilli undergoing pancreatic plus autolytic digestion. The next even numbered column shows the amount of N/20 acid liberated by 1 gram of the same mixture of tubercle bacilli undergoing autolytic digestion. The differences, not shown in the table, minus the correction for the added enzyme (last column) show the extent to which acid liberation was due to added pancreatic enzymes. Some of the strains had been used two years ago in the carbohydrate experiments. A number were very kindly furnished more recently by Dr. H. J. Corper of Denver. The titration of duplicate tubes was discontinued after the first few experiments, as close results were always obtained.

Results

The figures in table 2 may be read as follows, taking tubes F1 and F3 as examples (columns 9 and 10). Two similar 1-gram portions of tubercle bacilli of twenty-four days' growth were weighed into two graduated sterile 15-cc. centrifuge tubes. To each, 9 cc. chloroform Ringer solution were added plus 1 drop of phenol red indicator. Both suspensions were strongly acid. To tube F3, 0.7 cc. N/20 sodium hydroxide was added to bring the pH close to, but not exceeding 8. To tube F1, 50 mgm. trypsin were added followed by 1.1 cc. N/20 sodium hydroxide. The difference is the amount of sodium hydroxide required to neutralize the acidity of the trypsin. After two days' incubation both tubes were strongly acid. Tube F3 required 0.9 cc. and tube F1, 1.7 cc. N/20 sodium hydroxide for neutralization i.e., to change the reaction to pH 7.8. The difference between 1.7 and 0.9 cc.

indicates the probable liberation of acid from fat digestion. On the fourth day both tubes were acid again. Tube F3 was neutralized by adding 0.4 cc. N/20 sodium hydroxide; the total 1.3 cc. is recorded in table 2. Tube F1 required 1.3 cc.; the total 3.0 cc. is recorded. The differences between the tubes increased with increasing digestion period. After twenty-eight days, the total acid liberated in tube F3 by autolytic enzymes was 3.2 cc. The corresponding figure, 7.8 cc., for tube F1 is questionable because the titration end point was obscure. The end points remained sharp in tubes containing either tubercle bacilli or enzyme. In tubes containing both, the titration was difficult after about three weeks. The peculiar action of the indicator was frequently noted. The addition of a second or third drop of indicator merely provided a mixture of altered and unaltered indicator which still left the result in doubt.

One gram of tubercle bacilli undergoing autolysis for one month under the above described conditions, liberated on the average, 3.8 cc. N/20 acid. The corresponding figure was 6.5 cc. when 50 mgm. pancreatic enzymes had been added. Where did the acid come from? Part of the acid may be due to protein digestion. According to Sherman and Neun (1916), the digestion of 1 gram casein by 100 mgm. trypsin liberated 37.8 cc. N/10 acid in twenty hours. Pure wheat starch (Lehrman, 1930) when hydrolyzed yielded 0.95 per cent of fatty acids. To calculate the weight of fat digested is difficult in the absence of figures for molecular weights of true glycerides in tubercle bacillus. If the above figure, 6.5 cc. N/20 acid, be calculated to any probable mean molecular weight for fatty acids in tubercle bacillus, the result indicates that all or nearly all the fat was digested.

SWELLING OF TUBERCLE BACILLUS

In most of the fat digestion experiments, the suspensions were titrated in graduated centrifuge tubes for the purpose of noting any changes in the apparent volume of tubercle bacillus sediment. The following results are typical of many others.

In two tubes, 2.5-gram portions of a thirty days' growth of H37 were suspended in chloroform water in a total volume of 10 cc.

cators. If the experiment were performed without error, and the numerous corrections perfectly applied, the end result should have been four pairs of figures whose sums are the same, i.e., weights of soluble plus insoluble dry solids in 3.8 grams tubercle bacilli.

The data calculated to 100 grams of fresh bacilli are summarized in table 3. These indicate that autolytic enzymes alone brought one-fourth of the tubercle bacillus into solution in four days, and one-third in nine days. These proportions were only slightly increased by the added pancreatic enzymes. Although this is a preliminary experiment, it is difficult to avoid the conclusion that an appreciable part of the tubercle bacillus was rendered soluble. Limitations of the foregoing method are discussed in another publication (Berg, 1916).

SUMMARY

1. Tubercle bacilli undergoing autolysis *in vitro* liberated soluble digestion products. Among these were acids and reducing substances.
2. Tubercle bacilli undergoing pancreatic plus autolytic digestion *in vitro* liberated greater quantities of acids and reducing substances than by autolysis alone.
3. The swelling of tubercle bacilli in suspensions containing added pancreatic enzymes gave additional proof that digestion took place.
4. Autolytic enzymes alone brought one-fourth of the weight of tubercle bacilli into solution in four days, and one-third in nine days. These proportions were only slightly increased by added pancreatic enzymes.

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DISSOCIATION OF MYCOBACTERIUM LEPRAE[1]

G. B. REED

Department of Bacteriology, Queen's University, Kingston, Canada

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INTRODUCTION

In a recent series of papers from this laboratory the work of Petroff (1929) and his collaborators on the dissociation of tubercle bacilli has been confirmed and extended. It was shown by Reed and Rice (1931a) that a rapidly growing, avirulent strain of bovine tubercle bacilli when plated on suitable media produced entirely R type colonies but that after a series of generations on basic culture fluid, it was possible to isolate S colony types. In a study (1931b) of some twenty-eight additional cultures of tubercle bacilli from widely differing sources, including human, bovine and avian forms, it was found that recently isolated, highly virulent cultures and all cultures with a long history of persistent high virulence grew on such media as Petroff's gentian violet-egg in the form of S colonies. The cultures in the series which were avirulent or exhibited only low virulence, including those with a history of loss of pathogenicity, all grew in the form of R colonies. It was also shown (1931a, 1931b) that the S types of the human and bovine forms in fluids such as Sauton's or Proskauer and Beck's media grow principally in the form of a continuous spreading veil-like pellicle in contrast to the R types which grow in a rather heavier pellicle with a tendency to separate into island-like masses on the surface of the media. On the other hand, the avian S forms all grow in such fluids as a diffuse even suspension from top to bottom of the culture vessel while the avian

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R forms behave in much the same manner as the human and bovine R forms.

It was also demonstrated by Reed and Rice (1931) that correlated with colony structure and virulence, the R and S types differ in certain physical characteristics as measured by acid-agglutination and electrophoresis. They also differ antigenically, Rice (1931), as measured by complement fixation.

The present paper is concerned with a similar study of *Mycob. leprae.*

1. TWO COLONY TYPES IN CULTURES OF MYCOB. LEPRAE

A stock laboratory culture of *Mycob. leprae* when plated on Petroff's gentian violet-egg medium, and to a less definite degree on certain other media, exhibited two distinct colony forms and several intermediate forms. The colony regarded as R, in its most characteristic form, is a cone with an irregularly circular outline and sides sloping abruptly to a blunt peak. The surface is either granular or folded (plate 1, fig. 2). Many modifications appear, the most frequent being more flattened conical forms in which the base is relatively broader and more irregular in outline (plate 1, fig. 3). Occasionally the cone is so flattened that it appears almost a disk with very thin spreading and irregular outline (Plate 1, fig. 4). What is regarded as the S colony appears in contrast as a perfectly regular hemisphere with a smooth and waxy surface (plate 1, fig. 1).

In fluid media such as beef broth, Sauton's or Proskauer and Beck's synthetic fluid media, the R forms grow as a pellicle on a perfectly clear fluid, whereas the S grows first as a rather uniform diffuse suspension and as the culture matures there is generally a considerable accumulation of distinctly slimy sediment and frequently a rather mucilaginous surface pellicle.

A series of fourteen cultures regarded as *Mycob. leprae* were obtained from The National Type Culture and The American Type Culture Collections. As each culture was received it was plated on gentian violet-egg in such a way that several thousand well isolated colonies could be compared. In order to make an approximate catalogue of the colony form of the several cultures under examination all the colonies were considered as R or S, i.e., all intermediate colonies were listed as belonging to the type they most closely resembled. The results are shown in table 1.

All these strains, apart from the distribution of R and S colony forms appear strikingly similar in appearance on solid media, i.e., all the R types are similar and all the S are similar. There was, however, some difference in the degree of pigmentation and, as noted in a later section, there are considerable antigenic differences between the strains.

TABLE 1

Colony structure of 14 cultures of Mycob. leprae on the primary series of plate cultures

SOURCES	DESIGNATION	COLONIES ON PRIMARY PLATES
NCTC	No. 512, Clegg I	100 R to 1 S
NCTC	No. 513, Clegg II	100 R to 4 S
NCTC	No 509, Kedrowsky-Kraus	2,000 R to 1 S
NCTC	No. 514, Duval, chrome	100 R to 1 S
NCTC	No. 516, Bunckeroff I	All R
NCTC	No. 517, Bunckeroff II	100 R to 1 S
NCTC	No 518	All S
NCTC	No. 519, Barry	All R
NCTC	No. 522, Elly	All S
ATCC	No. 68	100 R to 1 S
ATCC	No. 69	1 R to 1,000 S
NCTC	No. 511, Nabarro Bayon	All S
NCTC	No. 521, Currie	All S
ATCC	No. 65	1,000 R to 1 S

Summary: 8 cultures, mixtures of R and S
4 cultures, all S
2 cultures, all R

Evidences of the origin and relationship of the types observed in the series of cultures were obtained from a study of variation and dissociation. Both the R and S forms have exhibited a considerable degree of stability on solid media whereas both types, but especially the S, have shown much variability when grown in suitable fluids to the extent of S to R and, less frequently, R to S dissociation.

2. S TO R DISSOCIATION

Dissociation of S to R was observed in three strains examined from this point of view. In each instance, stable S types were obtained from the cultures showing a mixture of types by subculturing from S colonies. The primary isolations at times produced pure S cultures, while in other instances repeated subculturing from single S colonies was necessary. Four to six generations of cultures showing only S forms were taken to indicate purity of type.

TABLE 2

S to R dissociation of Mycob. leprae No. 68

Four characteristic S colonies were isolated from the fourth culture showing only S and each inoculated into flasks of glycerol-infusion broth. Figures indicate the proportion of S and R types on egg plates made from the fourth successive 15-day culture in the broth.

	PROGENY OF COLONY NUMBER:			
	1	2	3	4
After four 15-day cultures in broth	100 S 10 R	100 S 500 R	100 S 1 R	All S

Experiments with culture 68

Quadruplicate cultures were made, from a single S colony on a plate culture of a stable S strain, in beef infusion glycerol broth and carried through four cultural generations each of fifteen to twenty days. Sufficient plates were made from the last broth cultures to permit comparison of several thousand colonies. The results shown in table 2 appear to indicate a pronounced dissociation of S to R during three generations of cultures in beef infusion glycerol broth. At the same time it indicates a difference in the variability of the progeny of apparently similar S colonies.

Experiments with culture 65

One S colony from the fifth consecutive culture showing only S was used to inoculate flasks of Sauton's fluid, Proskauer and

Beck's fluid and the same Proskauer and Beck's medium containing a heavy suspension of similar S organisms killed by ten minutes boiling. Plates were made at the end of four successive culture generations, each of twenty days. Table 3 summarises the results.

The fact that the Sauton's fluid[2] is very slightly buffered and becomes acid as growth progresses, in contrast to cultures in Proskauer and Beck's medium which remain neutral or become only slightly acid, suggested that the greater dissociation in the former medium might be the result of higher acidity. Dissociation of S to R has, however, been observed in other experiments in Proskauer and Beck's medium adjusted to a range of pH from 6.5 to 7.8; more acid or alkaline media appear to restrict variation but possibly only in proportion to the retardation of growth.

TABLE 3

S and R dissociation of Mycob leprae No. 65

Flasks of Sauton's fluid, Proskauer and Beck's fluid and Proskauer and Beck's with heat killed S were inoculated with S from the fifth culture showing only S. The figures indicate the results from serial cultures on the three fluids

FLUID MEDIA	COLONY FORM
1 generation in Sauton's	
2 generations in Sauton's	All S
3 generations in Sauton's	100 S to 1 R
4 generations in Sauton's	100 S to 6 R
1 generation in Proskauer and Beck's	
2 generations in Proskauer and Beck's	All S
3 generations in Proskauer and Beck's	All S
4 generations in Proskauer and Beck's	100 S to 2 R
1 generation in Proskauer and Beck's + dead S	
2 generations in Proskauer and Beck's + dead S	100 S to 10 R
3 generations in Proskauer and Beck's + dead S	100 S to 100 R
4 generations in Proskauer and Beck's + dead S	100 S to 1,000 R

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[2] The Sauton's fluid contains 0.5 gram Na_2HPO_4 per 1000 cc. (Compt. Rend. Acad. Sci., **155**, 1860, 1912), the Proskauer and Beck's fluid contains 5.0 grams Na_2HPO_4 per 1000 cc. (Baldwin, Petroff and Gardiner, *Tuberculosis*, Philadelphia, 1927, page 45).

The much greater dissociation in the medium containing dead S organisms is probably the result of a retarding action of the dead S on the similar type, a retardation which apparently does not extend to the R type.

Experiments with Clegg II (Culture 513)

From the eighth subculture of this strain showing only S types, twelve characteristic S colonies were isolated and inoculated, in two series, into flasks of Proskauer and Beck's fluid. At the end of two successive culture generations in the fluid, plates were made from the pellicle with results indicated in table 4.

TABLE 4

S to R dissociation of Mycob. leprae No. 513

Six characteristic S colonies were isolated from the eighth successive culture on solid media, showing only S, and introduced into 6 flasks of Proskauer and Beck's fluid and repeated in a second series. Figures indicate the results of plating from the fluid cultures.

	PROGENY OF COLONY NUMBER:					
	1	2	3	4	5	6
First series:						
After 7 days growth on Proskauer and Beck's	All S	100 S 1 R	All S	All S	All S	All S
After 1 culture of 7 days and one of 21 days on Proskauer and Beck's	100 S 1 R	100 S 2 R	All S	100 S 10 R	100 S 100 R	100 S 10 R
Second series:						
After 12 days growth on Proskauer and Beck's	All S	100 S 30 R	All S	100 S 30 R	All S	100 S 25 R
After 36 days growth in Proskauer and Beck's	100 S 200 R	100 S 200 R	100 S 200 R	100 S 300 R	100 S 100 R	100 S 400 R

The apparent difference in the results with the progeny of evidently similar S colonies may be in part the result of the sampling. As noted in the first section, the S grows in fluids as a diffuse suspension with some tendency, in mature cultures, to form a rather slimy surface pellicle whereas the R grows exclusively as a dry granular pellicle. Therefore in these fluid cultures of S suspected of containing R forms, the driest and most granular areas of the pellicle were always selected for subculture or for plating. Errors in this selection might account for the differences noted in the progeny of the different colonies. However, such differences as are indicated in table 4 have been so frequently observed that it seems more probable that the organisms making up the different colonies or the parent organisms of these colonies possess different potentialities of variability. At the same time it seems probable, from these results, that if a sufficiently long series of fluid cultures of an S is made, all will show dissociation to R.

3. R TO S DISSOCIATION

In all work on microbic dissociation the R types have been found more stable than S and there have been many reports on their complete stability; however, there are now many familiar cases of dissociation of characteristic R forms. A number of examples were recently quoted by Koser and Styron (1930) The several R cultures of *Mycob. leprae* examined in this study have behaved in a similar manner: all have exhibited a high degree of stability. A large number of R cultures have been carried through many generations without showing any tendency to dissociate while other apparently similar cultures have exhibited sufficient variability to make possible the isolation of intermediate and frankly S forms.

As in the former case, purity of an R type was considered established when four to six successive cultures produced only R's.

Experiments with culture 65

Seven characteristic R colonies were isolated from a well established R culture and inoculated into flasks of glycerol-beef infusion broth and each carried through four culture generations of seven to twenty days. In all of the broth cultures the principal growth consisted of a dry and granular surface pellicle, the usual R growth in fluids. In some, however, especially in mature cultures, the fluid under the pellicle became faintly cloudy, suggesting the S habit of growth. Accordingly in the successive transfers inoculum was removed from the depth of the fluid. As indicated in table 5, of the cultures from seven apparently similar R colonies, three remained throughout the four consecutive culture generations all R, whereas four exhibited marked R to S dissociation.

TABLE 5

R to S dissociation of Mycob. leprae No. 65

Seven R colonies, from the eighth culture of a series showing only R, were isolated and inoculated into glycerol beef infusions broth and transferred from the *bottom* for four consecutive cultures. The figures represent the proportion of R and S types on gentian violet-egg medium from each broth generation.

BROTH CULTURE	PROGENY OF COLONY NUMBER:						
	1	2	3	4	5	6	7
First, 15 days	All R	All R	All R	All R	All R	All R	All R
Second, 10 days	100 R 1 S	All R	100 R 2 S	All R	100 R 10 S	All R	All R
Third, 8 days	100 R 100 R	All R	100 R 50 S	All R	100 R 200 S	All R	All R
Fourth, 20 days	100 R 500 S	All R	100 R 200 S	All R	100 R	All R	100 R 1 S

TABLE 6

R to S dissociation of Mycob. leprae No. 513

From a well established R strain which produced only R's in a series of ten cultures on gentian violet-egg, ten colonies were transferred to Proskauer and Beck's fluid and egg plates made at the end of each fluid culture generation. The figures indicate the proportion of R and S in the plate cultures.

	PROGENY OF COLONY NUMBER:									
	1	2	3	4	5	6	7	8	9	10
After one fluid culture of 20 days	All R	100 R 1 S	All R	All R	All R	100 R 1 S	All R	All R	All R	All R
After two fluid cultures of 20 days each	All R	100 R 500 S	All R	All R	All R	100 R 100 S	All R	100 R 100 S	All R	100 R 1 S

Experiments with culture 513

From the tenth culture, producing only R types, ten characteristic colonies were isolated and each introduced into Proskauer and Beck's medium and carried through two consecutive cultures of twenty days each. The results shown in table 6 are in agreement with the observations on culture 65.

Several experiments have tested the influence of dead S and R organisms. In one, a single R colony, from the well established R series of the last experiment, was used to inoculate three series of Proskauer and Beck's fluid: one set containing a heavy suspension of boiled R organisms of the same strain as the inoculum, one set with a boiled suspension of S organisms and a control with no additions. Plates were made at the end of three successive generations grown in the fluids for twenty days each. Growth on the simple Proskauer and Beck's fluid and the fluid to which dead S organisms were added yielded only R colonies whereas the fluid to which dead R organisms were added exhibited an appreciable R to S dissociation. The very simple interpretation suggests itself that the presence of dead S organisms retarded the development of any S-like types which may have arisen and that the presence of dead R organisms is somewhat inhibitory to the development of R but not of S. Yet the R to S dissociation which has been observed in simple fluid media has been so irregular and so apparently dependent upon inherited factors that it is impossible to estimate accurately the influence of such external conditions.

Discussion of dissociation

No mention has been made of intermediate forms. They were, however, present in practically all plates made from dissociating cultures, and in some instances colonies could not be classified in the primary plates. Under such circumstances representative colonies were always isolated, emulsified and replated. The progeny generally consisted in part at least of characteristic S or R along with various intermediate types. The appearance of certain of the intermediate colony types is indicated in plate 1, figures 3 and 6. In no instance did any of the intermediates prove sufficiently stable to maintain themselves. It seems of considerable importance, however, that in a dissociating S culture: S, intermediate and R types may exist simultaneously. The figures mentioned in the tables are based on attempts to relate all colonies to the S or R either from the form or from breeding results.

It seems evident that although the S forms are relatively stable on solid media they readily dissociate in certain fluid cultures. In contrast, the R types are highly stable. At the same time it is significant that unmistakable R to S dissociations have been repeatedly observed. This is particularly important if observations on this species are to throw any light on the behavior of tubercle bacilli.

4. OXIDATION-REDUCTION POTENTIALS OF S AND R

It has been noted in previous sections that the S *Mycob. leprae* grow in fluid cultures as a uniform diffuse suspension in contrast to the R forms which always grow as a surface pellicle. This growth habit has been utilized in selecting variants in a dissociating culture. Assuming that the distribution of the two types may be the result of differences in oxygen requirements, conditions were made somewhat more extreme and the influence on dissociation examined. A series of Erlenmeyer flasks of 50 cc. capacity containing 40 cc. of Proskauer and Beck's medium was inoculated from a single R colony of a well established R strain of culture 513 and then sealed in jars in such a manner that there was from 50 to 600 cc. of atmospheric air above the surface of the fluid and a control open to the air through an ordinary cotton

TABLE 7

R to S dissociation of Mycob. leprae as influenced by the oxygen tension

Cultures of R organisms were incubated with varying volumes of air for 30 days and plated.

VOLUME OF AIR ABOVE CULTURE	PROPORTION OF R AND S IN 30-DAY CULTURES
cc	
50	100 R to 100 S
100	100 R to 1 S
150	100 R to 5 S
200	All R
600	All R
Open	All R

ERRATUM

In Volume XXIV, number 5, on pages 367 and 370, the curves, but not the figure numbers or legends, should be transposed.

plug. After thirty days growth gentian violet-egg plates were inoculated from the depth of the fluid cultures. The results stated in table 7 apparently indicate that a decreased oxygen tension favours R to S dissociation. This result might have been anticipated from the ordinary habits of growth of the two types in fluid media.

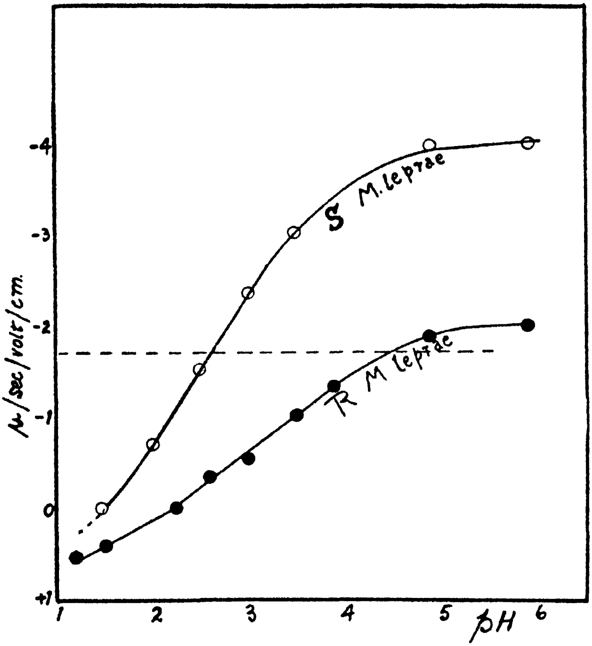

FIG. 1. CURVES INDICATING THE GROWTH-OXIDATION-REDUCTION POTENTIALS OF S AND R FORMS OF *Mycob. leprae* IN PROSKAUER AND BECK'S FLUID

Ordinates represent Eh in volts and the abscissa growth-time in days

These results suggest differences in the oxidation-reduction potentials. Accordingly, utilizing the technique previously described, Boyd and Reed (1931), determinations of the oxidation-reduction potentials have been made on cultures of both S and R *Mycob. leprae* growing in Proskauer and Beck's fluid.[3] A characteristic result, based on the average of several determinations is shown in figure 1. In cultures of the S type, showing diffuse growth, the potential decreased gradually throughout the growth period even after the sixteenth to eighteenth day when a thin pellicle had formed (shown by subculture to consist of S). With the R cultures on the other hand, the initial change in the potential was much longer delayed and only showed a marked decrease on the eighth or ninth day when a definite pellicle had formed; following this, the fall was more precipitous and more extreme than in the S cultures.

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[3] A more detailed consideration of oxidation-reduction potentials of growing cultures of several acid-fast species will be presented in a subsequent paper with E. M. Boyd.

5. ACID AGGLUTINATION AND ELECTROPHORETIC POTENTIAL OF S AND R[4]

In an earlier paper on the S and R types of tubercle bacilli by Reed and Rice (1931b), it was shown that the two types differ conspicuously in acid agglutination. This is in agreement with one of the earliest studies on bacterial dissociation, by DeKruif (1922).

The most casual observation of S and R types of *Mycob. leprae* suggests that they would behave in a similar manner; when the S are suspended in water or saline they readily form a rather stable suspension while the R types are suspended with difficulty and readily agglutinate or completely precipitate. For a more detailed examination, organisms grown in either fluid or solid media have been suspended in distilled water, washed three times and the washed suspensions added to series of Clark's phthalate-phosphate buffer mixtures. The tubes were incubated for two hours at 40°C. and read at once. The results with S and R types from several cultures are shown in table 8. Each set shown in the table is the average of four to eight determinations with different cultures. The age of the culture, the amount of washing and the length of time in distilled water slightly influenced the result but the variation was never more than 0.2 to 0.4 of a pH unit with the R and considerably less with the S forms. The R types agglutinate, as indicated in table 8 in solutions of pH 4.0 to 4.4 and the S in solutions of pH 2.5 to 3.0.

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[4] A more detailed account of these results in relation to data on other acid-fast species will be contained in a subsequent paper with G. B. Gardiner.

These results suggest differences in the potential of the two types as does the work of Kahn and Schwarzkopf (1931) with S and R types of tubercle bacilli. This has been tested experimentally with two procedures. In the first, the organisms from solid media have been suspended in distilled water, washed and the potential determined in the Northrop-Kunitz type of apparatus. An average set of results are shown in table 9. These indicate a definite difference in the potentials of the two types.

TABLE 8

Acid agglutinations of S and R types of Mycob. leprae

The + signs represent the extent of the agglutination.

ORGANISMS	pH OF BUFFER SOLUTIONS									
	2.5	2.8	3.0	3.2	3.6	4.0	4.4	5.0	6.0	7.0
S 513	++	++	–	–	–	–	–	–	–	–
S 65	++	++	–	–	–	–	–	–	–	–
S 509	++	–	–	–	–	–	–	–	–	–
S 516	++	+	–	–	–	–	–	–	–	–
S 517	++	–	–	–	–	–	–	–	–	–
R 513	++	++	++	++	++	++	–	–	–	–
R 65	++	++	++	++	++	++	–	–	–	–
R 512	++	++	++	++	++	+	–	–	–	–
R 519	++	++	++	++	++	++	±	–	–	–
R 521	++	++	++	++	++	++	+	–	–	–

TABLE 9

Electrophoretic potentials of S and R Mycob. leprae No. 513, washed and suspended in distilled water

CULTURE	S	R
	u/sec /volt/cm.	u/sec /volt/cm.
1	5.10	3.27
2	5.92	3.74
3	5.40	3.63
Average..............	5.28	3.55

In the second procedure the organisms were rapidly washed in distilled water and the thick washed suspensions added to a series of buffer solutions, approximately the procedure followed by McCutcheon, Mudd, Strumie and Lucké (1930). Figure 2

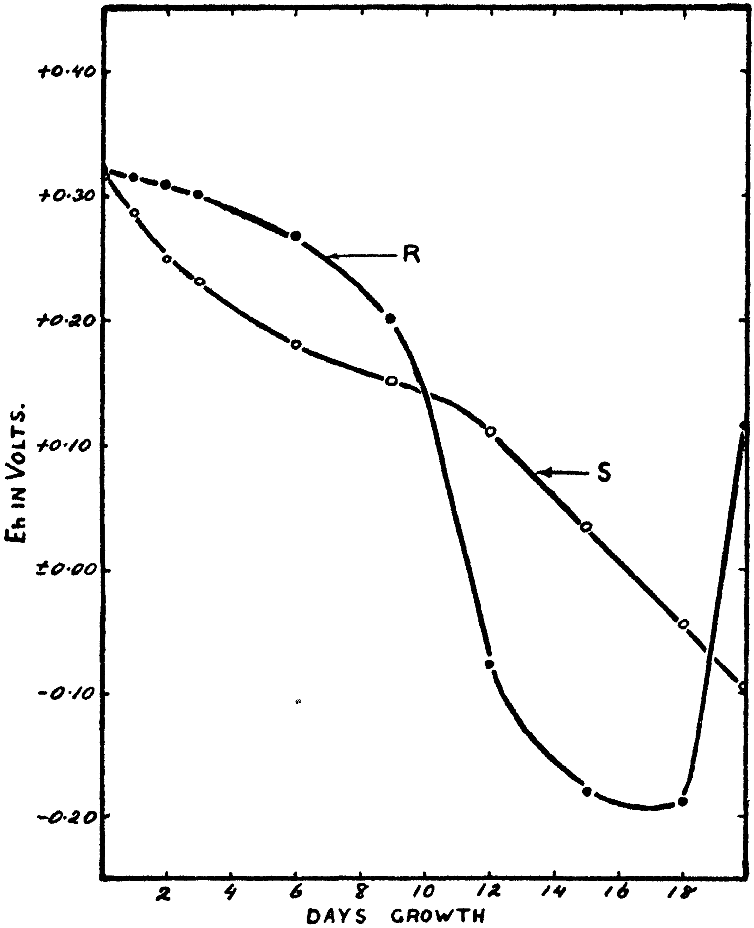

Fig. 2. Curves Indicating the Electrophoretic Potentials of S and R Forms of *Mycob. leprae* Suspended in a Series of Buffer Solutions

The horizontal dash line indicates the potential at which acid agglutination occurs.

indicates a characteristic result. The S organisms show a very low isoelectric point at approximately pH 1.5 and the R strains have their point at approximately pH 2.25. It is also apparent from the results indicated in figure 2 that when suspended in buffer solutions of pH 5 to 6 the differences between the electrophoretic potentials of the S and R types are slightly greater than when the two types are suspended in distilled water.

Comparing these results and the acid agglutination figures, it appears that where the potential of either type falls below approximately 1.5μ per second, per volt, per centimeter the organisms agglutinate. This is in approximate agreement with the conclusion of Northrop and De Kruif, Northrop (1928).

An S to R dissociation, it appears from these results, is accompanied by a physical change in the surface of the organisms. In the next section it will be shown that this dissociation is characterized by a loss in a specific S antigenic substance. There is no evidence to connect the two phenomena directly but the loss of the specific antigenic substance might conceivably alter the physical properties.

6. ANTIGENIC PROPERTIES OF S AND R

Complement fixation and agglutination reactions of S and R *Mycob. leprae* indicate quite as definite antigenic differences in the two types, as has been observed in dissociated forms of other species.

Complement fixation[5]

In an earlier paper from this laboratory, by Rice (1931), it was shown that an antigenic analysis of dissociated tubercle bacilli is possible by means of a quantitative complement fixation technique. Antisera prepared against S forms of mammalian tubercle bacilli were shown to fix complement in the presence of antigens prepared from corresponding S and R organisms and from other acid-fast species although the degree of fixation with the several types of antigens was conspicuously different.

Utilizing the same procedure, a series of antisera prepared against both S and R forms of *Mycob. leprae* were tested in the presence of aqueous extract antigens made from these two forms and from other acid-fast species. The results of this quantitative fixation precedure are shown in tables 10 and 11 where the average specific fixation of complement, by each combination of antisera and antigen, is indicated. Specific fixation is taken as the difference between the total amount of complement fixed, at 50 per cent hemolysis of the red cells, by immune rabbit serum plus antigen and the total amount fixed, at the same point of hemolysis, by the same antigen plus normal rabbit serum. The fixation index is arrived at by dividing the specific fixation with any combination of antisera and antigen by the fixation with the same serum and the homologous antigen.

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[5] Antigenic differences of dissociated types of several acid-fast species, based on complement fixation reactions, will be considered in more detail in a subsequent paper by Dr. Rice.

TABLE 10

Complement fixation results, Mycob. leprae S antiserum with homologous and other antigens

ANTISERUM	ANTIGEN	AVERAGE SPECIFIC FIXATION	X ANTIGEN FIXATION / HOMOLOGOUS S FIXATION
Leprae 513 S (1)	Leprae 513 S	0.0052	1.00
	Leprae 65 S	0.0049	0.96
	Leprae 513 R	0.0012	0.23
	Leprae 517 R	0.0009	0.17
	Human 13 S	0.0009	0.17
	Bovine 54 R	0.0004	0.08
	Avian Petroff R	0.0012	0.22
Leprae 513 S (2)	Leprae 513 S	0.0140	1.00
	Leprae 513 R	0.0108	0.77
	Leprae 517 R	0.0055	0.40
	Human 13 S	0.0072	0.51
	Bovine 599 R	0.0075	0.54
	Avian Petroff S	0.0089	0.64
	Avian Petroff R	0.0088	0.62

The figures of table 10 concerned with two samples of S antiserum indicate a relatively high fixation with homologous S antigens, a somewhat lower fixation with other S antigens and much lower results with R antigens of the same and other strains of *Mycob. leprae*; antigens made from other acid-fast species either R or S in type, give only slightly less fixation than with the leprae R antigens. On the other hand, as indicated in table 11, with anti R serum there is approximately the same fixation with the homologous R and with S antigens and again much less with antigens of other acid-fast species. In other terms the S antiserum appears to contain specific S antibodies, R antibodies and acid-fast group antibodies; the R antiserum lacks the S antibodies and contains R antibodies and acid-fast antibodies.

TABLE 11

Complement fixation results, Mycob. leprae R antiserum with homologous and other antigens

ANTISERUM	ANTIGEN	AVERAGE SPECIFIC FIXATION	X ANTIGEN FIXATION / HOMOLOGOUS SR FIXATION
Leprae 513 R (1)	Leprae 513 R	0 0136	1.00
	Leprae 517 R	0 0051	0 42
	Leprae 513 S	0 0122	0 90
	Leprae 65 S	0 0036	0.46
Leprae 513 R (2)	Leprae 513 R	0 0027	1 00
	Leprae 513 S	0 0029	1.07
Leprae 517 R (1)	Leprae 517 R	0 0056	1 00
	Leprae 513 R	0 0034	0 60
	Leprae 65 S	0 0060	1 07
	Human 13 S	0 0034	0 60
	Human 54 R	0 0025	0 45
	Avian Petroff R	0 0021	0 37
Leprae 517 R (2)	Leprae 517 R	0 0113	1.00
	Leprae 513 R	0 0066	0 58
	Leprae 513 S	0 0092	0 81
	Leprae 65 S	0 0063	0 56
	Human 13 S	0 0081	0.71
	Human 823 S	0 0056	0 50
	Bovine 599 R	0 0079	0 70
	Avian Petroff R	0 0083	0.73
	Avian Petroff S	0 0075	0 67

Serum agglutination[6]

Using a technique somewhat modified from that of Wilson (1925), a series of agglutination reactions has been carried out with the serum of rabbits immunized with heat killed emulsions of both R and S *Mycob. leprae* organisms and phenolized S and R antigens. The general results with leprae S antisera are indicated in table 12. With a No. 513 S antiserum, it may be noted, the homologous S organisms were agglutinated in a serum dilution of 1:1280, and S organisms from other cultures in slightly lower dilutions, whereas this serum agglutinated R organisms only in high concentration, or not at all, and acid-fast organisms of other species to approximately the same degree. Other S antisera reacted in a similar manner. Anti R sera, on the other hand, one instance of which is shown in table

TABLE 12

Agglutination results with anti M. Leprae 513 S serum and several antigens

ANTIGENS	DILUTION OF S 513 S ANTISERUM									
	1·10	1 20	1.40	1:80	1:160	1 320	1 640	1.1,280	1 2,540	Control
Leprae 513 S	+	+	+	+	+	+	+	+	–	–
Leprae 520 S	+	+	+	+	+	+	+	–	–	–
Leprae 516 S	+	+	+	+	+	+	+	–	–	–
Leprae 517 S	+	+	+	+	+	+	+	–	–	–
Leprae 513 R	+	–	–	–	–	–	–	–	–	–
Leprae 517 R	+	–	–	–	–	–	–	–	–	–
Leprae 509 R	–	–	–	–	–	–	–	–	–	–
Leprae 521 R	+	–	–	–	–	–	–	–	–	–
Phlei R	–	–	–	–	–	–	–	–	–	–
Avian S	+	–	–	–	–	–	–	–	–	–
Avian R	+	+	–	–	–	–	–	–	–	–
Human and bovine R and S	+	–	–	–	–	–	–	–	–	–

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[6] These results will be developed in more detail in contrast with tubercle bacilli and other acid-fast species in a subsequent paper with C. R. Lounsbury.

13, produced very little agglutination with homologous R organisms or with any other types.

TABLE 13

Agglutination results with anti M. leprae 513 R antiserum and various antigens

ANTIGENS	1.10	1·20	1.40	1.80	1.100	1 320	1 640	1 1,280	1 2,540	CONTROL
Leprae 513 R	+	–	–	–	–	–	–	–	–	–
Leprae 517 R	+	–	–	–	–	–	–	–	–	–
Leprae 509 R	–	–	–	–	–	–	–	–	–	–
Leprae 521 R	–	–	–	–	–	–	–	–	–	–
Leprae 513 S	+	–	–	–	–	–	–	–	–	–
Leprae 520 S	+	–	–	–	–	–	–	–	–	–
Leprae 516 S	–	–	–	–	–	–	–	–	–	–
Leprae 517 S	–	–	–	–	–	–	–	–	–	–
Phlei R	–	–	–	–	–	–	–	–	–	–
Avian R and S	–	–	–	–	–	–	–	–	–	–
Bovine R and S	–	–	–	–	–	–	–	–	–	–
Human R and S	+?	–	–	–	–	–	–	–	–	–

TABLE 14

Agglutination of M. leprae 513 S antiserum with M. leprae 513 S organisms before and after adsorption with a series of antigens

	DILUTION OF M LEPRAE S ANTISERUM								
	1 10	1 20	1 40	1.80	1 160	1 320	1 640	1 1,280	Control
Before adsorption	+	+	+	+	+	+	+	–	–
After adsorption with:									
M. leprae 513 S	+	+	+	–	–	–	–	–	–
M leprae 517 S	+	+	+	+	–	–	–	–	–
M. leprae 513 R	+	+	+	+	+	+	+	–	–
M. leprae 517 R	+	+	+	+	+	+	+	–	–
M. leprae 521 R	+	+	+	+	+	+	+	–	–

These results were confirmed with adsorption experiments. In a characteristic reaction indicated in table 14, the agglutinins of a 513 S antiserum were largely adsorbed with the homologous organisms and with S organisms from another culture whereas emulsions of different R cultures entirely failed to adsorb the S agglutinins.

These results indicate that the serum of rabbits immunized with *M. leprae* organisms of the S type contains, in rather high concentration, agglutinins which react with S antigens but do not react with R antigens. The S organisms must therefore possess a specific antigenic substance which is lacking in the R. At the same time the R-type does not appear to possess a specific R antigen but behaves in the presence of both S and R antiserum as do acid-fast bacteria of other species, suggesting, therefore, that its only antigenic content is a non-specific acid-fast group substance.

Discussion of the antigenic structure of S and R

From the antigenic analysis by both quantitative complement fixation and agglutination-absorption reactions it seems evident that the S to R dissociation of *Mycob. leprae* is accompanied by or is the result of a *loss* of specific S antigenic substance.

SUMMARY

1. It has been shown that many cultures of *Mycob. leprae* contain two or more colony types. In a group of fourteen cultures, regarded as belonging to the species, part were found to contain only S colony types, part only R types and part, mixtures of the two together with intermediate forms.

2. Both types have been shown to exhibit a considerable degree of stability on most solid media while both have dissociated in certain culture fluids.

3. S to R dissociation has occurred quite regularly on a variety of fluid media.

4. The R types under all conditions studied have appeared much more stable than the S, yet a sufficient number of instances have been recorded definitely to demonstrate R to S dissociation.

5. The S and R types were also shown to be distinguishable on the basis of growth habit in fluid media and oxidation-reduction potential growth curves, by acid agglutination, by electrophoretic potential and isoelectric points of suspensions and by antigenic content.

6. Antigenic analysis by quantitative complement fixation and agglutination-adsorption reactions have indicated that the S organisms possess a specific S antigenic substance which is lacking in the R organisms.

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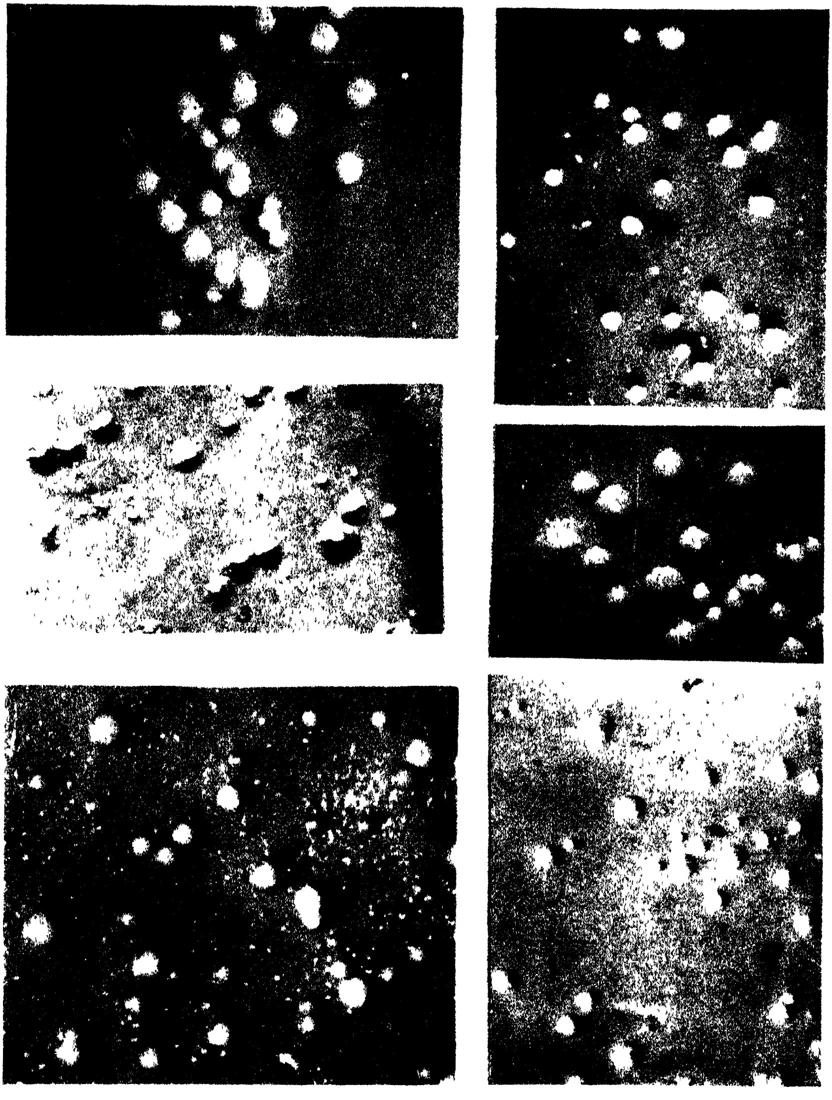
PLATE 1

PHOTOMICROGRAPHS OF COLONIES OF *Mycob. leprae* GROWING ON GENTIAN-VIOLET EGG MEDIA

1, typical S colonies; *2*, typical R colonies; *3*, flat form of R colonies; *4*, several typical S colonies and one flat R colony, a plate made from a culture in Proskauer and Beck's fluid showing S to R dissociation; *5*, several S and four typical R colonies from a source similar to that of *4*; *6*, intermediate types resembling S.



(G B Reed Dissociation of Mycobacterium leprae)

DISSOCIATION AND LIFE CYCLE OF BACILLUS MYCOIDES

I. M. LEWIS

Department of Botany and Bacteriology, The University of Texas

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INTRODUCTION

Bacillus mycoides was first described by Flügge in 1886. Since that time it has been the subject of numerous investigations and there are few species of non-pathogenic bacteria which are so well known. Our knowledge of its morphological and physiological characteristics is based principally on the excellent work of Gottheil (1901), Holzmüller (1909), Pringsheim (1924) and Nyberg (1927). Two studies have previously appeared on the subject of dissociation and life cycle, but the results and conclusions are so divergent that still further investigation seems to be necessary.

Oesterle and Stahl (1930) studied three typical strains which were subjected to various experimental conditions, including cultivation in unfavorable liquid media and exposure to light or ultraviolet rays. They obtained a great variety of new forms which bore little or no resemblance to the original type and which in the absence of further study would have been assigned to other species, genera, or even families. Some of these were isolated by direct plating methods, from cultures which had been incubated for long periods, while others were derived from the filtrates of such cultures. The list includes chromogenic cocci both red and yellow, small Gram-negative, and large Gram-positive bacilli. Some of the variants differed from the type form in motility, absence of spores, and failure to liquefy gelatin. In some cases the variants proved to be stable but, more generally, they reverted to the original when cultivated on agar. Reversion occurred from certain segments only of the agar colonies. As a result of these experiments, the authors believed they had proved the occurrence of a definite orderly life cycle including a filterable phase and several intermediate cyclostages between this and the original type. They described also marked pleomorphism of the original cultures, adopting the terminology of Löhnis and Smith (1916). Small spherical bodies, gonidia, and large yeast-like cocci, gonidiangia were the most prominent cellular elements present in aged cultures. The matrix was designated as symplasm. It is seen that the results and conclusions are in strict accord with the earlier work of Löhnis (1923) and Haag (1927).

Nyberg (1927, 1929) studied one strain in detail as to morphological characteristics and several additional strains as to dissociation. His observations and conclusions fail to confirm the findings of Oesterle and Stahl. He noted the same pleomorphism in all old cultures but was not able to conclude that any of the cell types, except the typical rods, reproduced without change in form when transplanted to fresh media. He found no support for Enderlein's (1925) theory of cell cyclogeny, or for the symplastic method of reproduction proposed by Löhnis (1916). Among the variant colonies which Nyberg obtained from old broth cultures, there were some which resembled the original types but others which were more like the well-known *B. vulgatus*. In some cases, the new forms proved to be quite stable, but in others they reverted to the original or produced still further variants. He concluded that the typical form which occurs in nature is a hybrid from which new types may arise by the usual method of segregation.

It should be noted that the experimental methods followed in the above investigations are not comparable. Nyberg employed ordinary nutrient solutions from which, by plating out on agar, he obtained the variable types. The method of Oesterle and Stahl was based on the use of unfavorable media or other harmful influences, and long periods of incubation followed by filtration. It does not seem reasonable to believe, however, that such widely divergent results and conclusions can be explained by the different methods employed.

The present investigation is concerned with the questions of pleomorphism, colonial variation, cell cyclogeny, methods of reproduction, and the presence or absence of a filterable phase in the life cycle. It has extended over a period of more than two years and involved the study of four typical strains under various experimental conditions.

TYPE STRAINS

The strains studied were isolated from soil by means of the usual dilution plate method. Single well-separated colonies of the typical spreading type were selected, from which transfers of a few threads were made to agar tubes. After sporulation occurred, dilution plates were prepared from heated spore suspensions. The final isolations were made by aid of a micromanipulator from single threads which had grown out over the surface. It is believed that this method is sufficiently reliable. Three strains isolated in this manner and one strain obtained from the American Type Culture Collection were included in the experiments.

Before beginning any experimental work, spore suspensions were prepared in neutral buffer solution and stored at low temperature to afford uniform material for inoculations. Dilution plates prepared at intervals from these suspensions failed to show any change in the original colony types.

All of these strains conform to the wide spreading adherent type with spirally arranged rhizoidal threads but obvious differences in the colonies may be noted. Strain A is characterized by rather fine threads in which the spirals turn always to the left (fig. 7). The remaining strains can be distinguished readily by the opposite spiral arrangement which also appears to be somewhat less symmetrical. The characteristics of strain B are shown in figures 1, 2 and 9. The threads are more robust and spread less rapidly over the agar. Strain C is shown in figure 8. The threads are long, slender and more inclined to become tangled together. The American Type Culture is not shown in the illustrations but it resembles closely strain B. It can be distinguished by less spreading growth on agar and by a slight difference in color which, in this case, is bluish while strain B is chalk white.

DISSOCIATION IN BROTH

Although many methods have been described for inducing dissociation, it appears that cultivation in liquid media, either in large volumes or by serial transplants in smaller volumes, has been found the most satisfactory. This method was employed for the preliminary experiment. The medium was plain extract peptone broth of standard formula, reaction pH 7.2. Flasks containing 300 cc. and large test tubes containing 30 cc. were inoculated from cultures which had grown for twenty-four hours on nutrient agar. The cultures were incubated at a temperature of 30°C. From each of the tube cultures, serial transplants were made to fresh broth at intervals of twenty-four hours. Transfers from tube to tube were made with a straight needle after the seed tube had been shaken thoroughly and then allowed to settle for one hour. Poured dilution plates were prepared from each tube of a series at the time of transfer to the next tube. The flasks remained one week before plating. They were then shaken thoroughly, permitted to settle for one hour, and plated by the dilution method.

Dissociation occurred promptly, both in the serially transplanted tubes and in the flasks. During the first twenty-four-hour period, no dissociation occurred, since typical colonies only were recovered from any of the first tubes. It may be noted also that the growth in these tubes was characteristic. It consisted of a tangled thread-like mass deposited at the bottom with no clouding of the broth. When shaken up, the growth settled back quickly to the bottom and the upper layers were practically clear. Microscopic examination showed very long chains of rod-like, Gram-positive cells. The second tube of each series gave some variant colonies which could be distinguished from the type form without difficulty. A typical example is shown in figure 2. It may be seen that the variant type resembles the parent strain B in the right hand spiral arrangement but is more compact.

Other types of variant colonies are shown in plates 1 and 2. These will be described in detail in a subsequent section. It suffices to note here that all connections between the typical colony form and that of such well known colony types as *B. subtilis* and *B. vulgatus* may be observed. The growth in these tubes appeared to be breaking up into shorter chains which gave a more uniform cloudiness to the broth after standing. Microscopic examination of material from the top layer revealed no differences in size, shape or staining reaction of the cells but a striking difference in length of the chains which now consisted of but a few elements.

Further serial transplants served only to cause more complete dissociation until, at the end of the tenth day, no typical colonies could be obtained from any of the four strains. The cells were not transformed into different types but remained in the original form, so far as could be determined by size, shape, and staining reaction. Plates from the flasks after two weeks incubation showed about equal numbers of typical and variant colonies when plated immediately after shaking. Although some striking changes in morphology were observed in the cells from all flasks, but not from the tubes, the variant colonies from the two sources appeared to be identical.

It appears from these results that dissociation occurs during a phase of rapid growth and that cultures which are not transplanted serially consist of a mixture of original and variant types. Transplanting in series after sedimentation serves to select the variant types by purely mechanical means and to afford optimum conditions for further dissociation which eventually becomes complete.

Although much has been done previously on the factors which influence dissociation it appears to me that this phase of the subject is by no means finished. Certain results which are to be presented later conflict with previous reports and in some cases an explanation for all observed phenomena is difficult if not impossible.

DISSOCIATION ON AGAR

It has been quite generally observed that dissociation occurs more readily in liquid media than on agar, although cases of the latter are known. In general, the strains of *B. mycoides* are quite stable on standard 2.0 per cent agar even on serial cultivation with twenty-four-hour intervals. Agar cultures stored at low temperatures after forty-eight to seventy-two hours incubation at 30°C. have remained typical for long periods of time. The American Type Culture No. 80 when received at this laboratory showed typical colonies only, when plated from water suspensions or young broth cultures. This strain appears to have remained true to type for many years. Strain A of my type strains, at times has shown a tendency to become less spreading with slight loss of symmetry. When carried through a series of twenty-five serial transplants on 2.0 per cent standard extract agar no further variation had occurred.

Giant colonies on petri dishes have failed to show either sector or marginal dissociation. Strain C has at times given rise to numerous secondary colonies (fig. 24). This phenomenon was observed by Nyberg (1929) in one of his type strains. The secondary colonies appear in cultures about two weeks old as small hemispherical knobs growing up out of the old threads or as deeper colonies lying embedded in the agar. They reach a maximum size of about 2 mm. in diameter although they are generally smaller. No secondary colonies have ever been observed in other strains when growing on plain extract peptone agar.

The subject of secondary colonies has received a great deal of attention since they were first reported by Günther (1895). It has been quite generally believed that secondary colonies denote dissociation and various theories have been advocated to explain their origin. Preisz (1904) seems to have proved that they originate in cultures of *B. anthracis* by the germination of spores. Both Stewart (1927) and Enderlein (1924) explain such colony formation as in some way connected with a sexual process. According to Enderlein, fusion of "spermit" and "oit" produces the fertile cell from which the colony originates. Stewart (1927) proposed a theory of sexual reproduction based on autogamy by which he explained not only the origin of secondary colonies but the cessation of growth of the parent colony as well. Hadley (1931) cites a case of secondary colony formation in *B. typhi* in which the origin was from gonidia or cells derived from gonidia. According to this view gonidia are produced only at certain stages of ontogeny when the cells have reached "reproductive maturity."

If this stage is reached in a liquid medium before plating out on agar, colonies of both the original and the so-called "G" type may appear, depending on the ability of the latter to grow on agar. But, if this state is reached among the cells of a colony on agar, then secondary colonies may arise from the gonidia. In an earlier report Hadley (1927) referred to a case of secondary colony formation in which the smooth parent colony gave rise to a rough variant form. The formation of secondary colonies on media containing fermentable carbon compounds which are not attacked by the parent strain has long been known and has been variously interpreted.

In the light of previous investigations, it seemed quite probable that the secondary colonies of *B. mycoides* might be identical with the more extreme variants obtained from broth cultures. Subsequent investigation failed to confirm this expectation. It should be noted that the secondary colonies in this case are obviously not of the "G" type since the cells invariably showed no marked variation from the normal rod. In old colonies there appeared to be more spiral rods than in the primary colony and spore formation was generally less abundant. Dilution plates were prepared from more than two hundred of the secondary colonies. A bit of the colony was taken up on the tip of a straight needle and suspended in broth from which the plates were then prepared. The colonies which developed on these plates were without exception identical with the original form. To say that strains isolated from the daughter colonies do not differ in any particular from the parent type might be questioned, but that the visible characteristics both as to colony form and morphology are identical there is no doubt. It appears that the problem of secondary colonies has not received the attention which it merits. The observations of Hadley seem to be based largely on work reported to him by others, while the conclusions of both Stewart and Enderlein are based on the uncertain hypothesis of sexual methods of reproduction. It is obvious from the results here recorded that secondary colony formation is not necessarily due to dissociation. It seems more probable that, with some modifications, the early explanation of Preisz is sufficient to account for the phenomenon in many cases and, particularly, in the absence of fermentable compounds from the culture medium.

DISSOCIATION UNDER ABNORMAL CONDITIONS FOR GROWTH

It has been quite generally held that conditions unfavorable to growth favor dissociation. Oesterle (1930) gave as the reason for not employing the usual nutrient media that they guarantee the highest constancy in form. In accordance with this principle many investigators have worked with media containing inhibitory substances of various kinds or have assumed that variant forms obtained from aged cultures were due to metabolic products. The principle appears to be well established that certain substances influence the process in some way but in many cases sufficient controls in favorable media have not been included in the experiments. In a recent paper by Hadley and others (1931) it was shown that dissociation of *B. dysenteriae* Shiga to the "G" type occurred in broth with, or without, the addition of lithium chloride. On the other hand it appears that no method for obtaining rhamnose-splitting strains of *Eberth. typhi* is known except cultivation on media which contain rhamnose. It is reasonable to suppose that the constant action of a substance favorable or unfavorable to a given metabolic function might lead to more or less permanent modifications.

However, it seems impossible to predict such effects and they must be determined experimentally for each organism. In the experiments to be reported here I have attempted to determine the effect of such influences as hydrogen ion concentration, temperature, metabolic products, culture media of low and high nutritive value and inhibitory chemicals.

The influence of temperature was found to be strongly marked. Flasks containing 300 cc. of standard broth pH 7.2 were inoculated and stored in triplicate at temperatures of 18°, 25° and 30°C. Dissociation occurred promptly at each of the higher temperatures. At the end of one week the number of variant and typical colonies was about equal on plates poured immediately after shaking the flasks. At the low temperature no variant colonies were obtained. The remaining flasks of this series were plated after an incubation period of four and six weeks. Not more than 5 to 10 per cent of dissociation occurred with the longest period of incubation at 18°C.

Initial reaction of the culture medium does not appear to be an important factor. *B. mycoides* grows best at a reaction of about pH 7.4. Growth is markedly inhibited at pH 8.8 and again at 5.4. In flasks of broth adjusted to initial reactions of pH 5.4, 7.4 and 8.8 no marked difference could be determined after two weeks incubation at 30°C. This result might have been caused by ability to change the reaction to a point where dissociation occurred. This was tested more fully for the acid range by the use of broth containing glucose. In flasks containing plain extract broth plus glucose 0.5 per cent and dipotassium phosphate 0.5 per cent, initial pH 5.8, the reaction remained acid throughout, with a final value of pH 5.4 at the end of two weeks. Dissociation at this time was no less marked than in control flasks with initial reaction of pH 7.4.

The rate of dissociation has been found to vary in culture media of high and low nutritive value. The several strains grow very well in standard broth diluted five, ten or even twenty times with distilled water. Dissociation occurred in dilutions of five and ten times but not when diluted twenty times. The flasks were tested after a prolonged period of incubation extending to four months but no variant type colonies could be observed. Cultivation in serial tubes was without effect. None of the strains produce variant types in a series of twenty-five tubes. Similar results were obtained in Heyden's nahrstoff medium containing 3.5 per cent dissolved in distilled water. Dissociation occurred in milk but at a much slower rate than in nutrient broth. Dissociation occurred in casein digest medium, yeast extract broth, nutrient gelatin and in 1.0 per cent of peptone or beef extract alone dissolved in distilled water. The addition of sucrose, glucose, lactose or maltose had no appreciable effect. These experiments indicate that depleted culture media are not favorable to dissociation. The effect of metabolic products on dissociation has been regarded as an important factor. Although the general question of isoinhibitory products has been studied extensively, differences of opinion have been expressed by Rahn (1906), Henrici (1928), Nadson and Adamovic (1910), and others. But it is well known that certain species such as *Pseudomonas fluorescens*, *Serratia marcescens* and *B. vulgatus* cause marked inhibition of other species.

The effect of its own metabolic products on *B. mycoides* was studied by Nadson and Adamovic (1910). The term dissociation had not come into use at that time and it appears that their results should be reinterpreted in the light of our present knowledge. They reported that old gelatin cultures which had been sterilized by heat, reinforced with fresh nutritive materials, adjusted to proper reaction, and solidified with agar gave colonies which resembled an actinomycete. They cultivated such strains for several generations without obtaining reversion. But similar strains may also be obtained from normal growth cultures as was shown by Nyberg (1929) and in the present study (fig. 15).

In the experiments to determine effect of metabolic products *B. mycoides* strains B and C, *Pseudomonas fluorescens*, *Serratia marcescens* and a strain of *B. subtilis* which was previously known to be inhibitory were employed. Broth cultures of these in shallow layers were incubated for a period of thirty days at room temperature, filtered through filter paper, and sterilized at 15 pounds pressure. None of the strains of *B. mycoides* proved capable of growth in the *Ps. fluorescens* or *B. subtilis* broth and all were inhibited in the presence of 5 per cent of the former or 25 per cent of the latter added to fresh nutrient broth. Dissociation occurred but required a longer period of incubation than in control flasks containing plain broth. At the end of three weeks not more than 10 per cent of the colonies were of variant types. Similar results were obtained in the case of an aged culture of *Serratia marcescens*. In broth containing its own metabolic products without an addition of fresh nutrients, growth was inhibited and dissociation occurred at a slower rate. When restored to full nutritive value by addition of peptone and beef extract, growth was as vigorous and dissociation as rapid as in control flasks of nutrient broth.

A somewhat similar experiment was conducted in which sodium chloride 4.0 per cent, lithium chloride 0.25 per cent, brucine sulphate 0.5 per cent, morphine sulphate 0.5 per cent, cocaine hydrochloride 0.125 per cent, and mercuric chloride 0.002 per cent were added separately to plain extract peptone broth. Inhibition of growth was marked in all cases except with lithium chloride and morphine sulphate. In these two cases growth was normal and was accompanied by rapid dissociation, while the cultures in which growth was retarded gave also a slower rate of dissociation.

It is recognized that this is a complex problem and that no general conclusions should be drawn from such limited experiments. Attention in these experiments has been given especially to the rate of dissociation during an incubation period extending over a maximum period of not more than six weeks and generally less. It is also true that change in colony form was the sole standard applied for determining dissociation in these and all preceding experiments. The results therefore are not to be compared with those of earlier workers who have employed various chemicals successfully for the purpose of causing changes in physiological processes or pathogenicity. Other species such as pathogens and more active fermenters are much better suited to such investigation than the one being considered here. The evidence which I have obtained points to the conclusion that changes in the colony form of *B. mycoides* depend primarily on normal growth conditions.

COLONIAL VARIATIONS

Because of its unique colony form *B. mycoides* is an especially satisfactory species for the study of colonial variations. The convenient terminology which has come into general use for denoting colony types as rough, smooth, phantom, intermediate or G is not very well suited for this species, but is applicable in a general way. The typical form is neither rough nor smooth in the sense which these terms are employed, while some of the more extreme variants could not be distinguished readily from the rough colonies of *B. subtilis.*

The type colony form to which all of the four strains conform has been described very well by Holzmüller (1909) and by Pringsheim (1924). The salient features of such a colony are adherence to the agar, root-like threads which spread rapidly over the surface, and the characteristic right or left hand spiral symmetry. By aid of the microscope the mode of colony formation may be readily followed on ordinary petri dish cultures. For this purpose it is best to make use of a 2.0 per cent agar containing not more than one half the standard amount of nutrients and in shallow layers. A 16 mm. objective reveals most of the details but higher objectives including oil immersion may be used to advantage. By selecting fields near the center of a giant colony and moving progressively to the extreme edge, one has an opportunity to observe clearly a colony of bacteria in the making. The same is true for many of the variant types.

The most striking feature in the development of such a typical colony is the extreme length of the cell chains and their growth parallel to each other to form the main threads. Figure 19, photographed with low magnification, shows the appearance of the main threads. They are smooth with no tendency to form loops or folds. As the individual chains of cells creep out from the main growth the characteristic general bending to right or left is always evident but there is no tendency to form folds. Growth of a cell chain is intercalary and the tip cells appear to be literally pushed along. Even though no impediment is encountered, these long chains occasionally break at a sharp angle. The free ends then continue their course as two parallel chains. Because of the breaking at sharp angles and the absence of wide, open folding of the threads we may designate this as the breaking type.

Although much has been said in the past concerning smooth and rough colonies or various gradations denoted by a combination of the terms, accurate descriptions of such colonies are largely lacking. This is doubtless due to the fact that other species of bacteria are less favorable for studying the mode of development. However several types of colony development have been described by Graham-Smith (1910).

During the course of the experiments already recorded, variant colonies were picked from the many plate cultures and transferred to agar tubes. They were generally replated after a few days incubation to determine purity and then stored at low temperature for further study. The entire collection was finally plated out and examined in order to cull out duplicates. By this process the total number was eventually reduced from more than three hundred to fifty, but there were still many duplications. These were then classified into the several variant types which are to be described here.

Variant type I resembles the original type strain in general appearance but has departed from it in two of the important characteristics noted above, namely, non-adherence to the agar and slight folding of the threads which gives it the beaded appearance. This type has retained the characteristic spiral habit without loss of symmetry (figs. 3 and 20). The type has proved stable on agar for almost two years but dissociates rapidly in broth to give further new types. It appears to be a transient which is met less frequently than some other forms but was seen many times. Milk appeared to maintain this form better than any of the liquid media tested and it was more frequently seen in plates from milk flasks inoculated with original strains.

Variant type II resembles its parent strain in symmetry but is non-adherent and no longer thread like. Very young colonies are round but this condition is soon lost. The growth is mat-like, rather thick with folded surface (figs. 4, 5 and 22). Microscopic examination at the edge shows that the individual chains of cells are still long but they fold instead of growing out in a straight course. They are still arranged parallel to each other in the larger threads and may grow up from the surface of the agar. This is a true folding type with marked difference in appearance from the original breaking type.

Variant type III resembles the preceding type but is more spreading. The symmetrical character has become less evident (figs. 16 and 17). When started by spot inoculation the primary growth is mat-like with folded surface, remaining almost round during the first few hours. The threads then begin to spread and there is a superficial resemblance to the original type. But the growth is non-adherent and examination with the microscope shows folding rather than straight breaking threads (fig. 22).

Variant type IV is the true cumulus cloud-like type with regular thick mat when young but putting out appendages much like type II when older (fig. 6). The surface is folded and shows the beautiful cut glass effect which has often been referred to as a characteristic of rough colonies in other species. A bit of the edge of such a colony may be examined with any magnification. The chains of cells are very long. They may grow out straight for a considerable distance but eventually begin to fold and form miniature colonies (fig. 21). This appears to be the delta type of *B. mycoides* described by Holzmüller.

Variant type V is in every way similar to the preceding except in thickness of the mat. The whole colony is identical with the thin appendages and never shows more than scattered miniature colonies connected by finer threads (fig. 21). This appears to be similar to the phantom type described by Soule (1927) for *B. subtilis*.

Variant type VI differs from IV in the total absence of appendages (fig. 16). Very old colonies may put out a faintly growing fringe but are always markedly different from the preceding (fig. 18). The edge of such a colony as well as the surface shows the extreme folding type of growth which is characteristic of the rough colonies of *B. subtilis* or *B. vulgatus*.

Variant type VII resembles the preceding but may be distinguished from it by differences in size and appearance and especially when viewed with the microscope. Young colonies are relatively smooth with entire edge. The threads no longer produce the characteristic bow-like folds and are much shorter. The internal structure is marked by beautiful striations. With age, regeneration at the edge reverts to the folding condition in which a partial symmetry may be noted (fig. 13).

The variant forms thus far described appear to represent a distinct linear series, beginning with the wide spreading rhizoidal type which is symmetrically spiral and terminating with a relatively smooth type. Between the two extremes there are many intermediate forms which appear to bridge all gaps and to form a closely connected series of types. All of these variants resemble each other in the characteristic of non-adherence to the agar and failure to invade below its surface. That this is a fundamental departure upon which further variation depends seems probable. The loss of adherence is probably to be explained by a change in the nature of the cell membrane or by substances excreted from it. All of the variants are soft and somewhat mucoid and in some cases this property is pronounced. With loss of adherence the chains no longer break but form open folds. Whether the correlation is more than a superficial coincidence it is not possible to say. Neither does it appear possible to explain the change from the folding type represented by number VI to the relatively smooth short chain type number VII. These appear to be quite similar to the smooth and rough forms described by Soule (1927) for *B. subtilis*.

A second series of variant forms remains to be described. These resemble the parent type in adherence to the agar and may be conveniently designated as type Ia, etc. Variant Ia is thread-like but the filaments are less symmetrically arranged and the colony is greatly reduced in size. It resembles the published descriptions of *B. prausnitzii* with which it might easily be confused (fig. 14). Microscopic examination shows an internal structure not unlike the original and consisting of breaking rather than folding chains of cells.

Variant IIa resembles the above somewhat but is sufficiently different to be distinguished with ease. The young colony forms a dense adherent mat resembling figure 15 but without threads. After about twenty-four hours it begins to regenerate and in the end resembles closely an original colony with a mat at the center. It was thought possible for some time that this type of colony might be due to a mixture of type IIIa and the original but this was not found to be the case. It appears that Gottheil must have been dealing with a similar strain for he discussed the question of differences between the central and marginal portion of the colony.

Variant IIIa differs from the above in size and marginal growth.

Even in old colonies there is never more than the slightest outgrowth (fig. 15). The central mat remains small and almost invisible during the first twenty-four hours and never reaches a diameter of more than 0.5 cm.

Variant IVa is an exact duplicate of the above but with no thread-like outgrowth whatever. The colony is a raised tough adherent mass which might be identified as *B. adhaerens* and appears to be the culmination of this series.

In this group of variants it again appears that a given tendency is followed through from beginning to end. Whether either series is linear in origin it is impossible to say definitely but the evidence favors this theory. Experiments have shown that any of the types, except the smooth number VII may be further changed by serial cultivation in broth. But these cultures like cultures of the original may consist of a mixture of several types. In a medium, such as milk, where the process is slowed down this is more evident. In liquid media the change is always in the direction of the smooth type. The several types of the adherent series have given rise to types of the non-adherent series on serial cultivation with final culmination in the smooth type. That the non-adherent series is unbranched while the adherent one branches at any point into the other there appears to be little doubt.

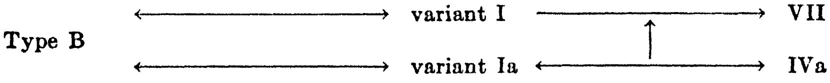
The results thus far reported appear to agree very well with those of some others who have worked with various species of the genus, such as Soule (1927) for *B. subtilis* and Nungester (1929) for *B. anthracis*. I have not, however, been able to obtain such extreme forms as were reported by Oesterle and Stahl (1930). Chromogenic and other cocci, Gram-negative bacilli and motile Gram-positive forms have remained conspicuously absent from all of the cultures. These forms have not been conscientiously avoided. They failed to appear.

Concerning reversion of colony types much has been written but there is probably no unanimity of opinion. I have no desire to enter into a discussion of this much debated question but merely to state briefly what I have seen and have not seen in the variant forms of this species.

Type III only, of the non-adherent series reverted at times to the original type. Such reversions were obtained fortuitously on agar plate cultures. Only certain segments of the colony reverted. I have seen no such reversion from any other type of this series nor has it been possible to obtain reversion by experimental methods. These included serial cultivation in liquid media of various formulae, cultivation on agar serially at 16°, 25° and 30°C. and isolation from the margin of colonies. It was thought possible that serial cultivation in liquid media might cause reversion since this has been shown to be true in some, but not all, species previously treated by others. The method of plating in this case was reversed by preparing plates immediately after shaking the tubes. Broth of standard formula, broth diluted twenty times, milk and nahrstoff were tested but no typical colonies were ever obtained in as many as fifty transplants. Cultivation on agar poor and rich in nutrients and varying amounts of agar as high as 4.5 per cent was without effect. The one method which has appeared to be most uniformly successful with other species, namely, cultivation in homologous immune serum, has not caused reversion thus far. This may have been due to the fact that a serum of high titer has not been obtained for the smoother strains.

All strains of the adherent series revert to the original when cultivated on standard nutrient agar at room temperature. Old agar cultures were generally found to consist of a mixture of the variant strains and the original. Types Ia–IVa could be kept true to type only by plating out and selecting a typical colony of the variant.

The sequence of all events may be seen in the accompanying diagram. It seems probable that the adherent joins the non-adherent at a point below the level of type I.



The most evident phenomenon of dissociation is the change from a thread-like to a mat-like habit with or without loss of adherence and symmetry of the spirals. The characteristics of the colonies are due to the post fission behavior rather than to visible changes in the morphology of the cells. In all cases the chains of cells growing on agar are long and growth is intercalary. This results in a certain amount of stress which may lead either to a break or to a folding loop. In the case of all non-adherent strains the latter occurs. That this behavior is due to changes in the cell membrane there seems little doubt. That some changes actually do occur is further supported by the characteristics of broth cultures. All looping strains break up into short filaments which give uniform cloudiness on shaking, while adherent strains form long chains which settle out leaving the broth practically clear. The relative cloudiness of broth cultures which have been shaken and settled affords a reliable index for determining the extent to which dissociation has occurred. This difference has been made use of as indicated above for hastening completion of the process by serial cultivation and for the isolation of variant strains. To obtain the adherent variants it is best to plate immediately after shaking the liquid cultures. The changes which lead to the formation of the compact adherent type are less obvious, but may be inferred from the variant forms which are still thread-like (fig. 14). Here it may be seen that the filaments of cells break instead of forming loops, but the main threads are no longer arranged in symmetrical spirals. The spiral arrangement of the threads has not as yet been satisfactorily explained although the subject has been extensively studied by Pringsheim (1924) and others. That this loss of symmetry is the chief factor in the formation of adherent variants seems to be obvious.

PHYSIOLOGIC VARIATION

To what extent changes in physiological processes are involved in the several variant forms has been studied experimentally. The four type strains and two strains each of the several variant types were included. Tests were made to determine liquefaction of gelation, effect on milk, and fermentation of the following compounds: sucrose, lactose, maltose, glucose, dextrin, salicin, glycerol, amygdalin, dulcitol, xylose, levulose and arbutin.

Slight differences in the rate of gelatin liquefaction were observed but all type strains and variants were found positive. The effect on milk appears to depend on its quality as has previously been shown by Nyberg (1929). In tubes prepared from market milk of unknown quality all strains caused complete coagulation without acid formation at the end of ten days. Peptonization, partial or complete, followed. Differences were not well marked. In a second test, fresh milk of high bacterial quality containing not more than 10,000 bacteria per cubic centimeter was used. In this case peptonization but no coagulation or acid formation occurred. These tests showed no significant differences between parent and variant strains but serve to emphasize the fact that coagulation of milk is dependent not on the bacteria alone but on the quality of the milk itself as well. This doubtless accounts for the fact that there are many conflicting reports in the literature on the non-acid coagulation of milk by various species.

The carbon compounds tested were sterilized separately in distilled water and added to plain extract peptone indicator broth in tubes. All type strains and their variants fermented the following compounds with acid only: sucrose, glucose, maltose, dextrin, levulose, salicin. No fermentation occurred in lactose, mannitol, dulcitol, amygdalin, arbutin or xylose.

These results seem to warrant the conclusion that no very profound changes in physiological characteristics have occurred. That variation in colony form may, in some cases, be accompanied by variation in physiological processes is not doubted, but that variation, either in form or function, may occur independently of each other appears to be equally well founded.

MORPHOLOGIC VARIATION

Due to the work of Gottheil (1901) and Holzmuller (1909) the morphology of *B. mycoides* is well enough known in a general way. However, new problems have since arisen based on details of cytology which their work did not include. Nyberg (1927) introduced several new terms including ectoplast and endoplast for certain granules, arthrospores for gonidia, monimospore for endospore, and planococcus for a very small comma-like motile cell which he suggested might be the same as the "spermit" of Enderlein (1925). Morphologic variation does not appear to have received much special study, although Oesterle and Stahl (1930), and Nyberg (1927) called attention to marked pleomorphism in all old cultures.

The recent studies of Henrici (1928) and of Hadley, Delves and Klimek (1931) have focused attention more sharply on this general subject. According to Henrici, three types of cells, embryonic, mature and sensecent may be recognized within a bacterial culture. These cells are distinguished by quite definite characteristics and each type is correlated with a growth phase. The transformation from embryonic condition through maturity to senescence is regarded as similar in principle to the cell differentiations which occur in multicellular organisms. The term cytomorphosis was chosen to designate the transformation of bacterial cells in cultures. This conception of morphologic variation does not appear to differ greatly from the general notion which already prevailed but the sequence of events is more definitely established. Hadley, Delves and Klimek (1931) criticised the method of Henrici and proposed a radically different explanation of morphologic variation based on cyclogenetic development. But the explanation of these authors appears to be little or nothing more than a restatement of the well known bacterial cyclogeny of Enderlein (1925). Differences of opinion will doubtless prevail but the issue appears to be clearly drawn between the advocates of cytomorphosis as developed by Henrici and the proponents of bacterial cyclogeny as developed by Enderlein and his followers.

The methods of Henrici are doubtless best suited for the accurate study of cytomorphosis but they are tedious and time consuming. It appears to me that the results already obtained by such precise means are adequate to establish the sequence of events within a bacterial culture and that facts of supplementary value may be determined by methods somewhat similar but less exacting. Accordingly I have made use of more conventional means of examination in this study. It has long been recognized that the study of bacterial cells *in situ* is a valuable method but it has not been very extensively applied to the study of variation. Of all the bacterial species, none is so well suited to such examination as *B. mycoides.* It is not necessary to prepare special microplate cultures since any petri dish culture may be studied. I have found no special advantages in microplates although they do give excellent preparations for study.

Examination of *B. mycoides in situ* reveals a most striking picture of morphologic variation. The cells grow out into extremely long chains which may be followed readily to the youngest tip. Embryonic cells are seen to be rather longer and more slender than older cells lying farther back within the colony. They contain few or no granules and stain intensely with the addition of dilute thionin, gentian violet, or fuchsin. Impression mounts stained by Gram's method show no reversion of the staining capacity. That these cells are morphologically and physiologically young there can be no doubt.

In older portions of the colony or even within a single long thread, cells may be noted which have become shortened and thickened. Measurements are not necessary to establish the difference. It is strikingly apparent. These cells invariably show numerous granules which have been designated as ectoplasts and endoplasts by Nyberg (1927). These are indicative of mature cells according to Henrici. On some culture media a great mortality occurs among cells of this type. This appears to be greater on media unfavorable to sporulation. Impression mounts or prepared films stained by Gram's method invariably show partial or complete reversal of the stain. Unequal staining is also observed *in situ* or in wet mounts stained with very dilute solutions.

The most notable feature of such mature cells, stained by vital methods, is the row of endoplasts lined up along the median axis. These are now seen to be clear refractile bodies, two to five in number. The precise nature of such bodies is not definitely known but they have been recognized by many workers in this and other species. Ruzicka (1907) believed that similar granules in *B. anthracis* consisted of prespore material and proposed the name "sporoids." Eisenberg (1909) regarded them as reserve lipoidal substance with a surrounding membrane of protein-like material, while similar bodies have been variously interpreted in *B. tuberculosis.* That such granules characterize mature cells rather than embryonic cells there is no doubt. They are always present in mature cells of *B. mycoides* and its variants but become more prominent when the medium contains either sugars or glycerol. In such cases sporulation is hindered and the granules become so prominent as to be easily confused with true endospores (fig. 34). At times the cytoplasmic content becomes reduced to mere bands lying at the poles or between the clear vacuole-like bodies. Rosen (1926) has described and illustrated a similar appearance in cells of *Phytomonas tumefaciens* with the suggestion that they resemble a mitotic figure.

A further feature which may be noted, both in embryonic and mature cells, when stained by vital methods is a deeply stained cap at either pole of the cell. These were described for *B. mycoides* by Amato (1909) who thought they consisted of nuclear material in the process of mitotic division. Similar observations were recorded by Mencl (1907) for *B. gammari.* The caps may be observed at times also in material stained by Gram's method. They appear to be the two new closing membrancs formed when a cell divides. The side membranes are not constricted but bound a colorless space between the daughter cells.

Under suitable conditions for sporulation the mature cells may become almost universally transformed to endospores, in which case no further morphologic variation occurs. But such general spore formation may fail, due either to intrinsic or extrinsic conditions. In this case senescent cells of various types occur. The term, senescent cells, will doubtless be rejected by many and is accepted here only with certain reservations.

The origin and subsequent behavior of these cells is the most critical point in the long controversy which has existed between monomorphists and pleomorphists. The term "senescent" does not appear to be objectionable when employed to denote the phase of growth or age of the cells themselves, but since it implies not only old age but approaching death some objections may be offered. It does not appear to have been shown conclusively that all such cells are senescent in the strict sense of the term. The old term, pleomorphic cells, appears to me preferable until such a time as more definite knowledge is available concerning their origin and function. It may be noted that such cells are frequently present in cultures long after the usual type of embryonic cell and the mature type have disappeared. In such cases they are often more like embryonic cells in structure and might well serve to carry on the life of the culture. The term "dauer cells" might not, therefore, be inapplicable in many cases.

Many mature cells die without transformation either into endosporangia or into any of the several pleomorphic types. Such is often a pronounced feature in this species. Pleomorphic cells are invariably present, at least in small numbers, in all old cultures but they are more numerous when spore formation is retarded or suppressed. They are therefore most abundant in agar cultures containing sugars or glycerol or in deep broth cultures where aeration is not suitable for spore formation. They may be observed *in situ* or in prepared films. Each method has some advantages.

The several types of pleomorphic cells are well known. Henrici (1928) recognized ten types in *B. coli*, Löhnis and Smith (1923) described six types in *Azotobacter*, while others have shown similar conditions for many species. But the precise origin of such cells has not been clearly observed in most cases and our conception of origin has been obtained by inference rather than by observation. The most clearly marked cases of pleomorphic cells in *B. mycoides* are the small or medium sized cocci frequently noted in other species, large yeast-like or oval formed cells, long rod-like cells larger than mature or embryonic cells, spiral forms, and myceloid cells. How such cells originate and how they function may now be considered.

There appears to be no great difficulty in regard to the smaller and medium sized coccus forms. They may arise either as terminal spherical buds or by repeated binary fission of the typical rod-like cells. In cases of the former method, buds may originate from cells which appear to be ordinary vegetative cells or from cells which are somewhat swollen. Such buds are indistinguishable from true cocci but because of their subsequent behavior I prefer to designate them as pseudococci (figs. 32 and 33). There is some evidence that such cells may themselves continue division within the old culture giving rise to chains of cocci but this is a difficult matter to determine with certainty (fig. 31). Pseudococci may arise also by binary fission. Such cells are generally recognized by the fact that they are never true spheres. At times they may be oval, much shorter than typical rods, with slightly rounded ends or they may be decidedly flattened on the contiguous surfaces. Long chains of such cells are not uncommon (fig. 33). I have seen no cases in which pseudococci arose by lateral buds, by formation within the mother cell or as regenerative bodies as described by Löhnis (1921).

All attempts to isolate strains of cocci from cultures in which such cells are abundant resulted in failure. They could not maintain this form in fresh media but invariably grew out into typical rods at once. These cells do not appear to be senescent in the sense the term is generally employed. They stain deeply with simple aqueous dyes and are strongly Gram-positive. Absence of granules and the staining capacity place them as more like true embryonic cells except in shape.

Giant yeast-like cells frequently predominate over all other types in old cultures. They have been noted in great abundance in many of the mucoid variant strains when grown on agar containing sucrose or glucose (fig. 36). They are likewise invariably produced in great numbers in very shallow layers of broth in flasks. These cells may become very large, almost spherical or somewhat elongated. At times such cells occur in pairs which seems to indicate a belated division (figs. 29 and 36). They may also form one or more bud-like cells (fig. 30). Obviously these are the cells which have been designated by Löhnis and others as "gonidiangia." They have also been designated as zygotes, asci, "oits" or chlamydospores. Like the pseudococci described above, they stain deeply by vital methods and are uniformly Gram-positive. I have seen no evidence of internal cell divisions or liberation of smaller bodies although many hundreds of such cells have been examined *in situ* as well as in wet mounts stained or unstained. So far as staining reaction and presence of granules might indicate, they have more the characteristics of embryonic than of mature cells. A bit of material containing such cells may be transferred from agar to fresh broth and examined at regular intervals to determine their behavior in fresh media. The beginning growth appears bud-like, not infrequently at either end, but the buds lengthen out to become typical rod-like cells. I have seen no evidence that these cells arise through cell fusions such as those described by Mellon (1925) for *B. coli* and by Potthoff (1924) for Chromatium. Neither do they appear to be "oits" as claimed by Enderlein (1925) for *Vibrio cholera.* My observations are in better accord with those of Bergstrand (1923) for *Corynebacterium diphtheriae.* If any special name is required for such cells, I would prefer the term, chlamydospore, of Bergstrand but this does not appear necessary to me and in some respects I regard it as objectionable.

The term, chlamydospore, has been used by mycologists to designate reproductive cells in which the differentiation is much more pronounced. The cell membrane has not been observed to thicken and the cell has no such definite characteristics as the well known chlamydospores. Further, I have not infrequently observed that such cells may function as belated endosporangia long after the period of general sporulation has passed. Such sporangia are easily recognized by their size and deep staining properties. A similar observation was made by Grohmann (1924) for other species. Thus it appears that they have not become truly differentiated cells. In very old cultures some of these cells become less deeply stained and more or less autolysed and now appear to be truly senescent in the sense that death is approaching or has already occurred. These appear to be the cysts or involution cells of some writers.

Greatly enlarged rod-like cells (fig. 35) and spiral cells (fig. 27) are less frequently met but are not uncommon in all aged cultures. *The very long cells appear to be due to failure of true cell division although the factors involved here are less certain. Even in* young broth cultures limiting lateral membranes are sometimes lacking. Holzmüller (1909) reported this as a notable feature of some strains. Spiral forms arise more frequently within the agar than upon its surface. The least frequent of all pleomorphic cells is a very long myceloid type. At times I have been inclined to doubt the purity of cultures in which such cells were observed. They are long, slender, about the diameter of typical rods and may be variously coiled resembling very fine hypha threads of a mold. But their occurrence in all strains has convinced me that they are pleomorphic cells of *B. mycoides.* I have not been able to cultivate any of these types.

A further type of cell formation which has been frequently described but which I have not been able to observe with certainty is by internal division of the mother cell which may be either a normal rod or a swollen cell designated as the gonidiangium. That the large yeast-like cells described above do not function in this manner in this species, I regard as certain. The material for examination has been abundant, the cells are large and repeated observations have been made. Neither have the so-called lateral buds attached to cells nor gemmules borne within vegetative cells been certainly identified.

That this is a most difficult point to determine definitely is generally recognized. The literature of this subject has been fully reviewed by Löhnis (1921) and more recently by Hadley, Delves and Klimek (1931). It may be seen from these reviews that the evidence is conflicting and much of it was taken from uncritical work of earlier periods. That various structures including artefacts, the various reserve granules, Gram-positive ectoplasm attached or detached from the surface, shrunken and balled up protoplasts have been confused with true conidia there appears but little reason to doubt. An examination of fixed films stained by Gram's method soon convinced me that conclusions based on this method of study are wholly without value. Examination of cells *in situ* or wet mounts stained by vital methods are less confusing but some of the same difficulties are encountered. There appears to be no evidence whatever that gonidia are formed in the embryonic cells. To search for them in such cells of *B. mycoides* is as fruitless as to search for endospores in the same group of cells. A single structure only might lead to erroneous conclusions. The polar caps described above take up the stain more deeply and might cause some confusion. As the cells approach the mature condition and especially when autolysis sets in the picture becomes more complicated and confusing. Between the now numerous colorless granules small bands of deeply stained cytoplasm, especially at the poles, might be regarded as gonidia. This is especially true in cultures grown on agar containing sugars or glycerol (fig. 34). But that any of these structures are true reproductive bodies borne within the cell is, to say the least, questionable. The so-called senescent or pleomorphic cells take up the stain deeply and fail to show the granules characteristic of the mature cells. I am obliged, therefore, to reach the same conclusion concerning them that has already been stated for embryonic and mature cells.

Hadley, Delves and Klimek (1931) have suggested that gonidia are formed in bacteria at certain stages only after the vegetative cells have reached "reproductive maturity," and could not therefore be observed except at certain favorable periods of ontogeny. According to these authors, such stages occur after long periods of vegetative activity, best provided by serial transplants in broth tubes. When the proper period of ontogenetic maturity has been reached, gonidia are formed and a new cyclostage is established. Formation of gonidia is not again to be expected until the complete cyclogeny of the species has occurred. This appears to be in strict accord with the broad outlines of the well known theory of bacterial cyclogeny proposed by Enderlein (1925) but lacking in the details of nuclear behavior. It would appear to cast some doubt on the earlier work in which gonidia seem to have been shown in any and all kinds of cultures.

A further question in regard to morphologic variation concerns the symplastic theory first proposed by Löhnis and Smith (1916). Search of the literature fails to reveal much evidence for or against this theory but such opinions as have been expressed appear to be about equally divided. It has been accepted by Enderlein (1925), Almquist (1922), Lieske (1926), and Meyn (1931) while the term has been used loosely by some writers to denote the zoogleal residue of old cultures without offering an opinion as to its real nature. No support was found by Nyberg (1927), Bergstrand (1923) or Rosen (1928).

In all old broth cultures of *B. mycoides* there is a copious somewhat slimy zoogleal deposit which persists for long periods of time. In cultures held at room temperature for more than a year, no very great changes could be noted. Examination of such material has invariably revealed the presence of cellular elements and transplants to fresh media have resulted in colonies of the original parent strain as well as of variant types.

Whether or not there is continued activity resulting in dissolution into an amorphous living mass, from which new cells are organized by a process of regeneration, it is impossible to determine with certainty from such material. The evidence appears to me highly doubtful. When agar cultures are examined *in situ* there seems but little doubt that such a process is wholly lacking. Old agar plate cultures have been examined after having been protected against drying out and held at room temperature for periods as long as six months. The spore chains may be observed lying in long parallel threads with no visible changes. Similarly the several types of pleomorphic cells noted above may be seen and, to all appearance, they are the identical cells formed during the active growth period. In one strain only, secondary colony formation occurred (fig. 24). No other signs of regenerative activity could be noted.

It is somewhat surprising that such an important and far reaching theory should have received such slight attention at the hands of bacteriologists and biologists in general. It is evident that Löhnis meant to include both fungi and protozoa as well as the bacteria in the group of symplasm-forming organisms. His language is unmistakable: "Sooner or later many cells of bacteria, fungi, and protozoa may dissolve, or according to a frequently used expression—autolysis may take place. This may mean death to the organisms but by no means always. If the observations are continued, new development may become visible in these amorphous residues, and new cells may be evolved similar to or different from those of the preceding generation" (Löhnis and Fred, 1923, p. 32). If such a method of cell regeneration from a formless mass of protoplasm can be shown in any of these groups of organisms it will then become necessary to revise the biological doctrine of cell continuity. While this is not offered as sufficient grounds for rejection of the theory it should point the way to caution in accepting it for cell continuity has appeared to be an established fact. The symplastic theory of cell disorganization and reorganization appears to me to be based on a confusion of the sequence of events in ordinary cytomorphosis, autolysis and death.

FILTERABILITY

Although some species of bacteria are known to be filterable in the ordinary vegetative condition, bacteriologists as a rule have been slow to accept the notion that a filterable stage occurs generally as a normal phase in a life cycle more complicated than we have been accustomed to accept. That such a phase might occur under some conditions, with or without the full cyclogenic significance attached to it by some writers seems not impossible. The recent experimental work of Kendall (1931), Sherman (1931), and Hadley, Delves and Klimek (1931) indicates that filterability may be a much more common characteristic of bacteria under certain conditions of cultivation than had hitherto been believed. But that all or even many species of bacteria are at times both filterable and characterized by a cyclogenic mode of development seems to be an assumption unwarranted by known facts.

B. mycoides has been cited by Hadley, Delves and Klimek (1931) as one of the three species for which such a complete cyclogenic ontogeny is definitely known. They referred to the earlier paper by Oesterle and Stahl (1930). The two additional species noted in this list were *Azotobacter*, Löhnis (1923), and *B. anthracis,* Haag (1927). So far as I have been able to ascertain none of these has been confirmed. Nungester (1929) reported failure to obtain filterability of *B. anthracis.*

So far as I am able to report from my experiments neither *B. mycoides* nor its variants have been found filterable. It seems unnecessary to report these experiments in detail. It may be said, however, that plain broth cultures of all ages from two weeks to as much as six months have been included. The series of filtration experiments included also the cultures grown in unfavorable media and reported in a previous section. The records show that a total of 55 flask cultures were filtered. In each case, the broth was first centrifuged until relatively clear and then passed through Berkefeld filters N, V or W with minimum negative pressure. The filtrates were distributed in tubes and held indefinitely at room temperature protected against evaporation. Frequent inspection showed no visible growth except in a relatively few tubes. In such cases the growth had every appearance of being due to chance contaminations. In the light of the most recent work these results might be explained as only apparently negative and recorded as such because of failure to recognize the faint almost imperceptible cloudiness which characterizes the filterable stage. But whether this is true or not, all efforts to obtain visible growth by subsequent cultivation from these tubes on agar or broth resulted in failure. Under the conditions of these experiments I am forced to conclude that neither *B. mycoides* nor the variant strains already described are filterable. The occurrence of such a filterable stage remains to be shown. It is of interest to note that similar organisms, *B. megatherium*, *B. vulgatus*, and *B. mesentericus*, have not been found filterable under sufficiently controlled conditions by Rettger and Gillespie (1932) and that Nungester (1929) found no filterable cells in *B. anthracis*.

THE LIFE CYCLE

The term, life cycle, has come into such general use that I venture to discuss it now in its application to *B. mycoides*. When colonial variation alone is considered, it has been shown that the transformations in form are orderly and that the sequences may be readily followed from the original basic type form toward a definite predetermined type with which the process terminates. The two extreme forms are connected by a complete series of intermediates. I think no one can view the evidence without accepting it thus far (plates 1 to 3). Such variation is obviously not in harmony with the well known principles of DeVries, Darwin or Mendel but is not unlike the method known as orthogenesis—continuous variation in a predetermined direction. Examples of such variation are not unknown but on the contrary are frequently met in such well known plants as the Boston sword fern, *Nephrolepsis exaltata-bostoniensis*. Thus far considered, the process of dissociation appears to meet the requirements of a true cyclogeny, but in the matter of reversion I have not been able to show such a well marked chain of events leading back in reverse order to the original basic form. Such reversions as have been noted, indicate a slight but not very definite tendency in this direction. The evidence, therefore, must be regarded as giving but doubtful support to cyclogeny as the term is generally employed.

When morphologic variation is considered, it is impossible to see any support whatever for the theory of cyclogeny. The cells of the original type strains and their variants are similar in shape, size, structure, and functions. There is no transformation, progressive and orderly or otherwise, from a basic cell type to cells of a different order which are capable of propagation in a pure state. Morphologic variation has been seen in all aged cultures but the variant cells failed to maintain their form in fresh culture media. Accordingly they cannot be regarded as cell types which have any special significance in reproduction, heredity or cyclogeny. Colonial variation and morphologic variation are not correlated. The differences in colony form are due to the manner of cell aggregation within the colony rather than to differences in the individual cells of which the colony is composed. The absence of a filterable stage, together with the observed facts of reversion, morphologic and colonial variation, force me to conclude that *B. mycoides* affords no support for the theory of a complex life history through which the organism must pass in a complete cycle of development.

In the present state of knowledge concerning a sexual method of reproduction in bacteria, it seems scarcely necessary to discuss Nyberg's theory that the variants of *B. mycoides* are due to hybrid segregation. It may suffice to say that I have seen no evidence of conjugation or other sexual method of reproduction upon which such a theory must depend. Moreover, the phenomena of dissociation are not what would be expected from such segregation. Dissociation in this species appears to be limited, almost if not entirely, to colonial polymorphism. Orthogenic variation with a relatively high degree of stability in the variant colony types affords the most satisfactory explanation of the phenomena observed.

SUMMARY

Previous studies on dissociation of *B. mycoides* have shown both pleomorphism and variant colony types. The results and conclusions, however, were not in complete harmony. In one case the variants were regarded as cyclostages in a complex life cycle while in the other they were thought to be due to segregation of a hybrid form.

Four typical strains have been studied as to dissociation, pleomorphism, filterability, and life cycle. The type strains showed minor differences as to colony characteristics but uniformity in morphological and physiological characteristics, and in the phenomena of dissociation.

Dissociation occurred in some but not all of the liquid media tested, but not on agar. The rate of dissociation depends on several factors. The most important of these were found to be the temperature of incubation and a culture medium suitable for rapid growth. The rate was generally retarded by depleted media, metabolic products, low temperature, inhibitory chemicals, and media poor in nutrients.

The typical colony is unique in form and any departures however slight may be observed by direct microscopic examination. The characteristic features are adherence to the agar, long parallel filaments of cells which aggregate to form rhizoid-like threads, and a more or less regular spiral symmetry. The chains of cells never form loops or folds but break at a sharp angle.

Two series of variants were found to occur in all the type strains. These may be designated as adherent or non-adherent. In either case there is progressive orderly variation in one direction. The variants are relatively stable on agar but not in broth.

Non-adherent variants failed to revert to the original type while such reversion occurred in all adherent strains when cultivated on agar. Non-adherence is always correlated with looping rather than breaking of the threads at sharp angles. All variants and the type strains were found to be similar in morphological and physiological characteristics.

Pleomorphism is a marked feature in all aged cultures. The variant types of cells are not regarded as specialized reproductive cells either asexual or sexual. No convincing evidence could be found to support the theory of reproduction by means of gonidia, symplasm, or conjugation. This conclusion is based principally on the results of direct examination of the growth *in situ*. All attempts to obtain growth from the filtrates of liquid cultures resulted in failure.

The evidence in favor of a cyclogenetic mode of development is mostly negative. Colonial polymorphism is regarded as due to orthogenetic variation. The environmental conditions under which variation occurs as well as the sequence of events and a possible mechanism have been discussed.

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PLATE 1

All illustrations figs. 1 to 36 were prepared from cultures which grew in standard broth or on standard agar containing 2.0 per cent of agar.

FIG. 1. Type strain B at end of twenty-four hours. ×3.5.

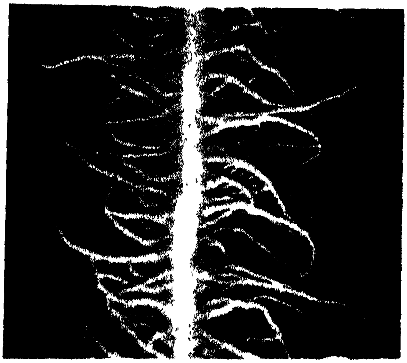
FIG. 2. Type strain B dilution plate from second tube of series after a sedimentation period of one hour. ×1.

FIG. 3. Variant type I from type strain B showing beaded or nodose habit and right-hand spiral symmetry. ×3.5.

FIG. 4. Variant type II from type strain B. ×3.5.

FIG. 5. Variant type II from a pure culture about one year after original isolation. ×1.

FIG. 6. Variant type IV from type B. ×3.5.



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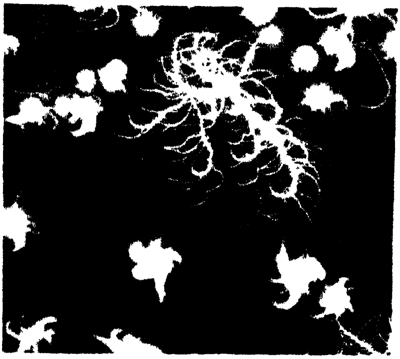

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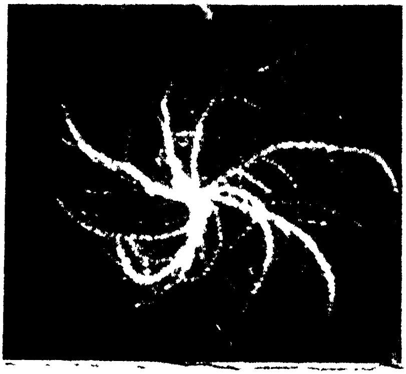

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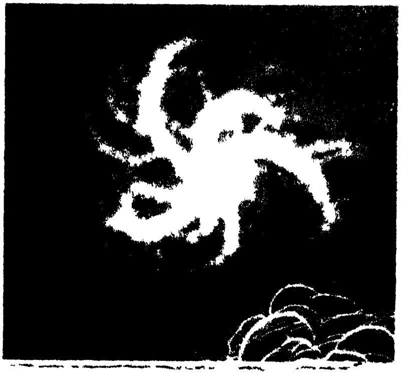

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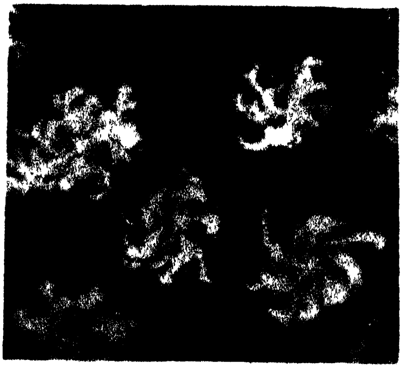

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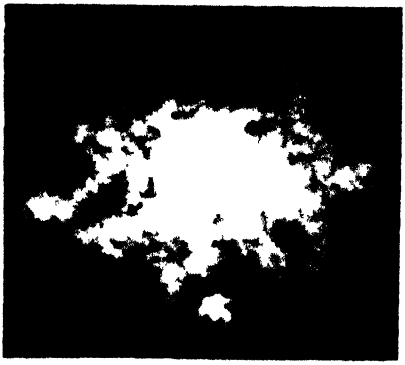

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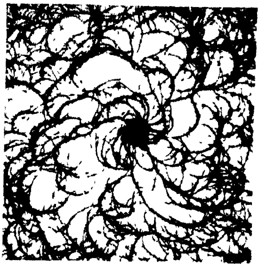
(I. M. Lewis: Dissociation and life cycle of *B. mycoides*)

PLATE 2

Fig. 7. Type strain A with left hand spiral symmetry. ×1.5.
Fig. 8. Type strain C with right hand spiral symmetry. ×1.5
Fig. 9. Type B with right hand symmetry and more robust threads. ×1.5
Fig. 10. Variant type III from strain A. Giant colony. ×1
Fig. 11. Variant type IIIa from type strain C. ×3.5
Fig. 12. Variant type IIIa from type strain B. ×3.5
Fig. 13. Variant type VII old colony showing smooth type with folding type at margin. ×1
Fig. 14. Variant type Ia from type strain A. ×1.
Fig. 15. Variant type IIIa from type strain B. ×1
Fig. 16. Variant type VI from type strain B. ×1.5
Fig. 17. Variant type III from type strain A showing giant colony with mat like center and pseudo threads. ×1
Fig. 18. Variant type IVa at end of two weeks. ×1



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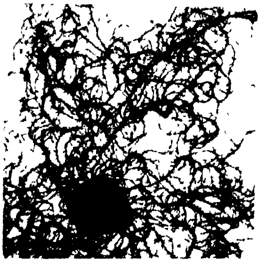

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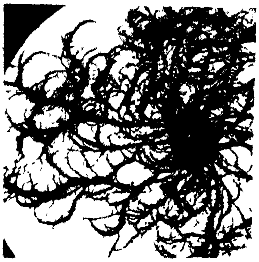

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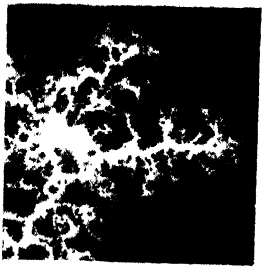

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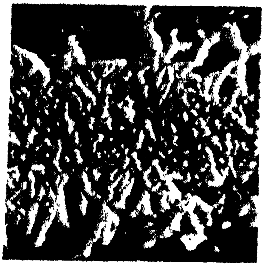

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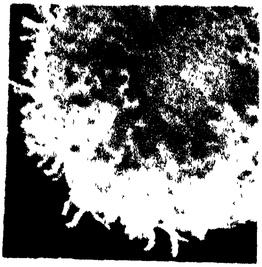

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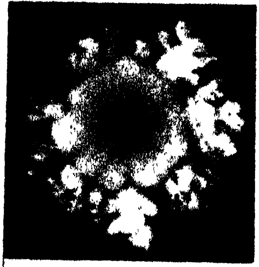

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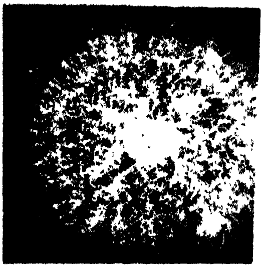

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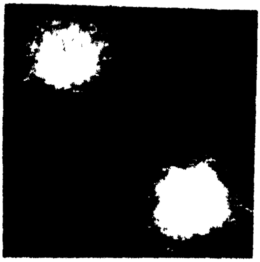

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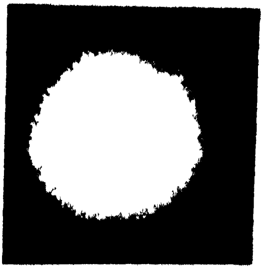

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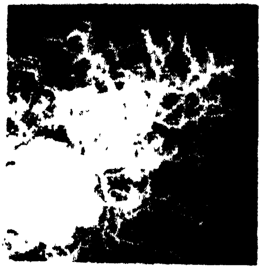

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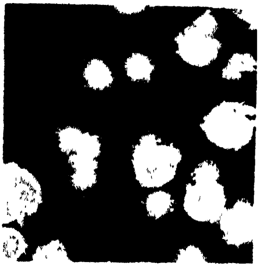

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(I. M Lewis. Dissociation and life cycle of *B mycoides*)

PLATE 3

Fig 19 Type strain A showing typical breaking type with smooth unfolded threads ×30

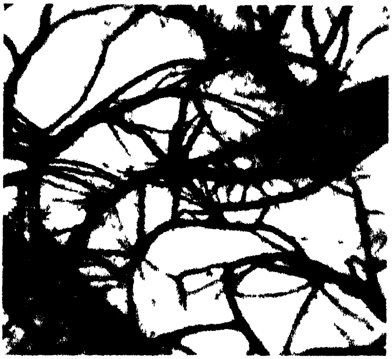
Fig 20 Variant type I from type strain B with nodes which are caused by folding ×30

Fig 21 Variant type IV from type strain B with looping threads which form miniature colonies and appendages ×30

Fig 22 Variant type with more compact folding habit ×30

Fig 23 Variant type VI from type strain B with compact folding habit and no appendages ×30

Fig 24 Daughter colonies formed on agar colony after two weeks No lysis of the original threads can be noted Type strain C ×3 5



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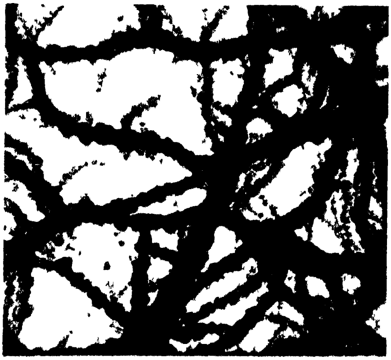

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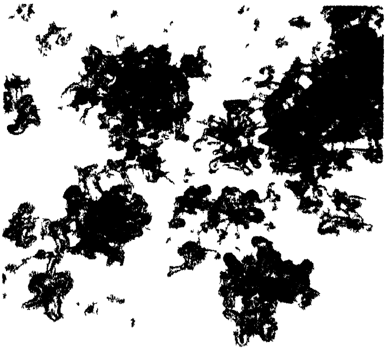

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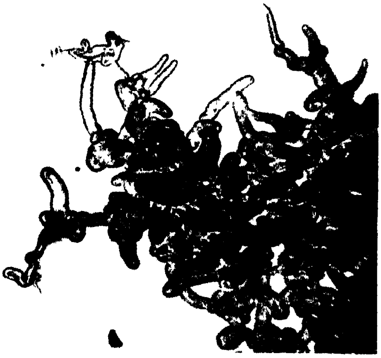

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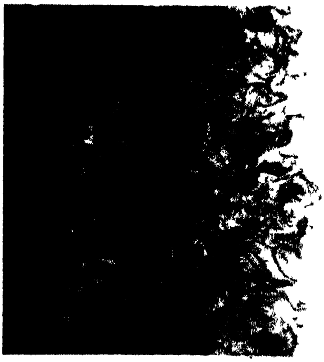

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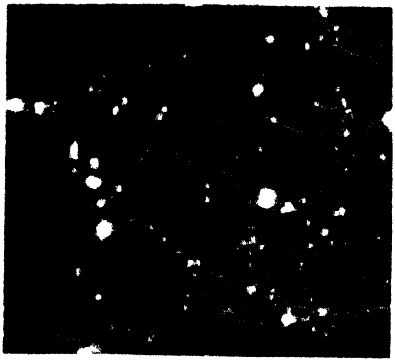

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(I M Lewis, Dissociation and life cycle of *B mycoides*)

PLATE 4

Unless otherwise noted the figures were made from cultures of type strain B stained by Gram's method All magnifications are the same, about 1100

FIG 25. Typical rods in chains from twenty-four-hour agar culture

FIG. 26. Endospores from agar cultures seventy-two hours old on medium containing one-half standard amount of peptone and beef extract

FIG. 27. Broth culture at end of seventy-two hours Spiral cells, reduction in size, and autolysis are shown.

FIG. 28 Broth culture at end of seven days showing typical rods, pseudococci and autolysis

FIG 29 Glucose agar culture at end of ten days showing typical endospores, one large endosporangium and giant cells

FIG 30 Same as figure 29 with large pseudococci

FIG 31 Formation of pseudococci in old broth culture

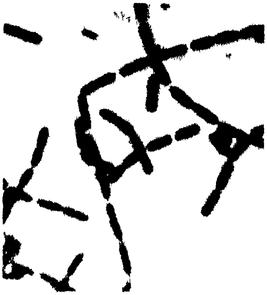
FIG 32 Agar culture showing endospores and formation of pseudococci by budding

FIG 33 Pseudococci formed by binary fission Broth culture of type strain A

FIG 34 Glucose agar culture at end of seventy-two hours showing large endoplasts unstained and bands of cytoplasm stained deeply The stained cytoplasm is not regarded as gonidia

FIG 35 Glucose agar culture seventy-two hours showing pseudococci, elongated rods and autolysis

FIG 36 Shallow broth culture showing giant pseudococci, autolysis and budding Type strain A.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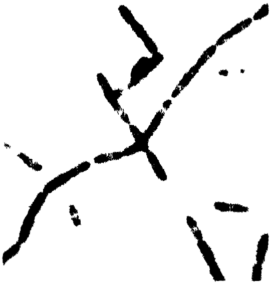
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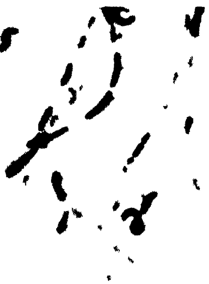
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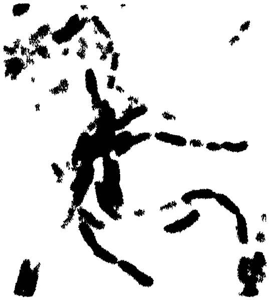
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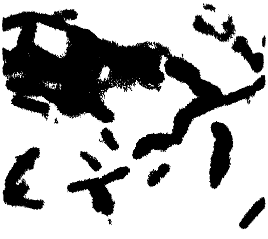
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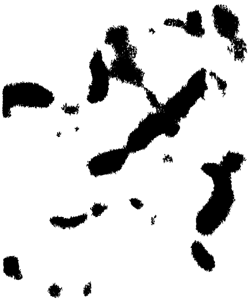
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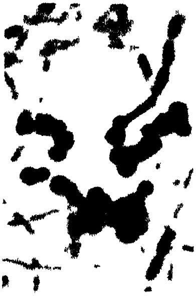
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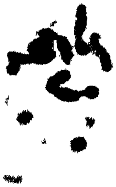
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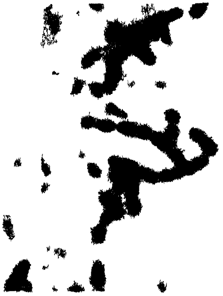
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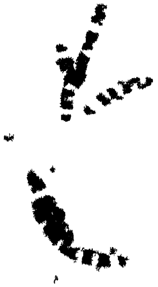
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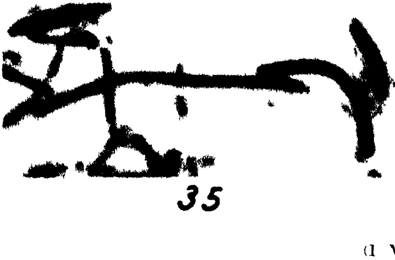
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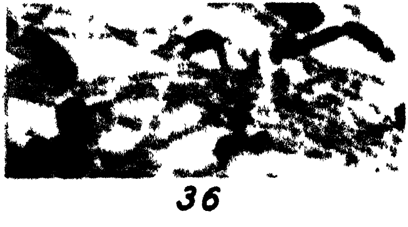
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(I. M. Lewis: Dissociation and life cycle of *B. mycoides*)

LETHAL EFFECT OF ALTERNATING CURRENT ON YEAST CELLS[1]

RALPH L. TRACY, JR.

Fruit Products Laboratory, University of California

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It is well known that the passage of a strong electrical current through cell suspensions exerts a killing effect. It is believed that this bactericidal action is primarily due to the formation of toxic compounds in the solution by direct current and the generation of sufficient heat in the solution by alternating current to produce lethal temperatures. However, few data have been reported on the effect of alternating currents applied to cell suspensions at non-lethal temperatures. The studies reported in this paper have been made under this latter condition.

Cohn and Mendelsohn (1879) reported that they were unable to sterilize a suspension of *B. prodigiosus* in a nutrient solution by passage of a direct current for twelve to twenty-four hours. Further growth of the bacteria in the treated solution was arrested, owing to the formation of toxic compounds by electrolysis, but upon transforming small quantities of the treated media and organisms to fresh nutrient solutions normal growth occurred. No attempt was made to determine quantitatively whether the number of living cells was reduced during the passage of the current.

Fermi (1891) bacteriologically examined the sewage sterilizing process devised by Webster (1891) in which iron electrodes were used and found that passage of a current of 0.5 to 1 ampere through the sewage water reduced the viable cell content to 1 to 2 per cent.

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[1] This investigation was made possible through funds granted by the California Committee on the Relation of Electricity to Agriculture, to whom the writer wishes to express his thanks. The work was conducted under the general direction of Professor W. V. Cruess and Professor B. D. Moses of the College of Agriculture of the University of California.

He believed that the germicidal action was due to the formation, coagulation and precipitation of iron hydroxide, which carried the organisms with it in settling.

Spikler and Gottstein (1891) obtained reductions in the numbers of *B. prodigiosus* and other microörganisms by passage of an induced current through suspensions in distilled water for twenty-four hours at temperatures below 30°C. They believed the germicidal action was primarily electrical since no metallic electrodes were used in direct contact with the media.

Stone (1909) found that a direct current of 0.1 to 0.3 milliampere stimulated growth of bacteria and yeast until lethal concentrations of toxic compounds, from electrolytic action on the zinc and copper electrodes, were formed in the solution. Application of electrical charges from a static machine accelerated or decreased the number of living cells, according to the severity of the treatment. In none of the experiments, however, was sterilization obtained.

Thornton (1912) obtained marked killing action on *B. typhosus* and other bacteria with an 80 cycle electrical current at 65 volts. The electrodes were of platinum and the temperatures of the solutions were maintained between 55° and 65°C. Although the temperatures had some lethal action upon the microörganisms, he believed that the electricity was directly active in producing the killing effects, primarily because it was found necessary to maintain a current density and voltage gradient above a minimum to obtain any kills. With direct current, the killing action was due to the formation of toxic compounds.

Beattie and Lewis (1931 and 1920) pasteurized milk in a continuous stream by an alternating current, 80 cycles at 4000 volts. The milk flowed between copper electrodes in the treatment chamber and during the period of exposure reached 64°C. They believed the killing effect was not alone dependent upon temperature, because of the short time of exposure and the necessity of maintaining a current density and voltage above a minimum.

Finkelstein and Anderson working with the "Electropure" apparatus reported (1919) that pasteurization was probably due to the heat generated within the media by passage of the current.

Kleiber (1925) was able to kill yeast suspended in beer wort or in grape juice by direct and alternating currents. The killing effect was due to the formation of toxic compounds by direct current and to the generation of lethal temperatures by alternating current.

Fritz (1929) obtained a decided killing effect upon yeast with a 220 volt, 50 cycle, current. Platinum and silver electrodes were used. He believed the silver salts and free chlorine generated by electrolytic action were responsible, because at 3,000,000 cycles, where electrolysis is negligible, there was little killing effect.

Devereaux (1929) and later Gelpi and Devereaux (1930) found that the electropure process reduced the numbers of *B. anthracis* spores in milk to a much greater extent than did the holding method.

Tracy (1931) reported that yeast suspended in fruit juice could be killed by the electropure process within sixty seconds at temperatures 2° to 3°C. below their thermal death point.

Semichon (1931) sterilized fruit juices by the "Matzka" process. The liquid flowed between silver and aluminum electrodes in a thin film and reached 55° to 65°C. during the period of exposure. Matzka believes the self-induced electrical current generated from contact of the juice and metals is, among other factors, responsible for the killing effect.

METHOD

The source of electricity was a 120 volt, 60 cycle current. Voltage and amperage were varied for experimental purposes by means of two rheostats in series with the electrode chamber. The voltmeter and ammeter were connected to the leads of the electrode chamber.

Suspensions of yeast cells were subjected to action of the current between hard carbon and graphite electrodes in a small pyrex glass chamber. The chamber was made in the form of a T-tube, the two horizontal arms holding the electrodes while the side arm, placed vertically, served as a means of introducing and removing samples of media in the chamber. The chamber is shown in diagram 1.

Two of these tubes were used. In one, the inside diameter of the horizontal tube was 1.1 cm.; in the other, 2.5 cm. The vertical tube in each case had an inside diameter of 0.4 cm. In several tests a third chamber for ellipsoidal electrodes 1.17 cm.^{2} in area was used. The surface of a single electrode in the tube 1.1 cm. in diameter was 0.95 cm.^{2} in area, and in the tube 2.5 cm. in diameter, was 4.9 cm.^{2} in area.

The electrodes were sealed in the chambers with Dennison's number 2 sealing wax. This was accomplished by filling the grooves at the head of the electrodes with soft wax. When the wax cooled, the electrode surfaces were sterilized in a flame and the rods immediately inserted into the T-tubes and adjusted to the desired distance apart. A flame was then played on the glass over the wax until the electrodes were properly sealed to the glass. Care was taken not to smear the electrode surfaces with the molten wax. In most of the tests, the distance between the electrode surfaces was 5 to 7 mm.

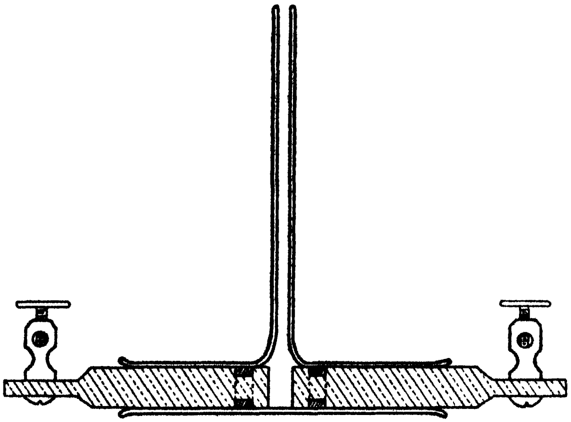

DIAGRAM 1. CROSS SECTION OF ELECTRICAL CHAMBER

Pyrex glass chamber, continuous double lines. Carbon electrodes, alternate dash and straight lines. Grooves in the electrodes filled with wax, small heavy straight lines. Posts for electrical connections at the end of each carbon electrode. Chamber proper for treating solutions lies between the carbon electrodes directly under the vertical tubulature. The volume of this chamber is variable.

The cell was externally cooled by a rapid stream of cold water. The temperatures of the treated solutions were measured by a small thermocouple and potentiometer, and only the highest temperatures attained were recorded. The thermocouple was inserted into the solution through the vertical tubulation.

Cultures of a "fine grained" or "non-agglomerating" strain of *Saccharomyces ellipsoideus* yeast between three and fourteen days old were used. These age limits were selected because it was found that twenty-four hour cultures were most susceptible to heat, while three to twenty-one day old cultures were of greater, but approximately equal, resistance. The cultures were grown upon 2 per cent agar preparations of concentrated white grape juice diluted to 20 per cent solids.

Before making a test, the chamber was filled with sterile grape juice, connected into the circuit and the current adjusted to the proper amperage and voltage by means of the rheostats. The actual potential drop across the solution was slightly less than the apparent potential drop as recorded on the voltmeter because the voltmeter was connected in parallel between the electrode leads instead of directly to the electrode surfaces touching the solution. This deviation from the true voltage across the solution was extremely small, as the resistances of the copper wire leads and the relatively large carbon electrodes were practically negligible. This solution was then removed and replaced with freshly inoculated grape juice upon which the test was made. After treatment, samples of the solution were removed, diluted from 1:10 to 1:1000 in sterile distilled water and plated on grape juice agar. When the electrodes of 1.1 cm. diameter were used, the quantity of the samples was limited to 0.1 cc., but with the larger electrodes 1 and 0.1 cc. samples were taken. Counts were made after seventy-two to ninety-six hours' incubation at 32° to 34°C. Control samples of 0.1 cc. volume were taken from the solution before treatment and treated similarly except that they were diluted from 1:100 to 1:10,000.

EXPERIMENTAL

Thermal death point

The thermal death point of the yeast in grape juice was determined in order that it might be known whether or not lethal

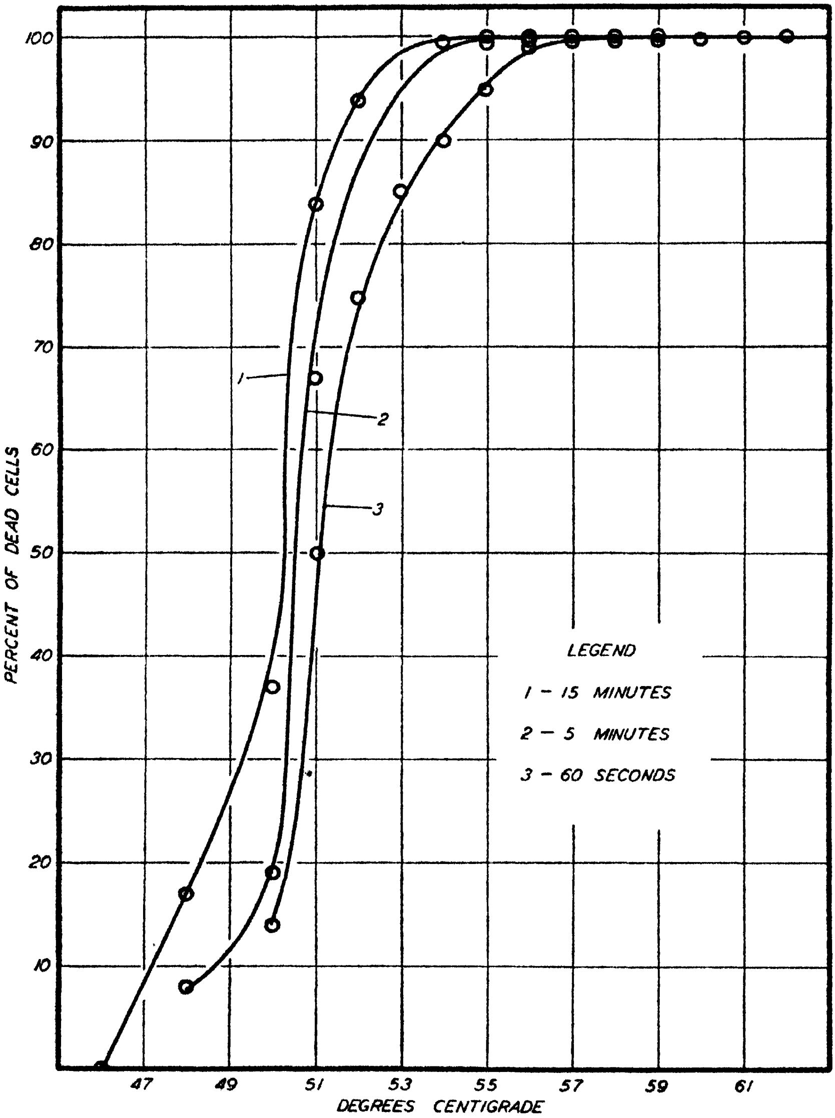

FIG. 1. KILLING EFFECT OF VARIOUS TEMPERATURES ON *Saccharomyces ellipsoideus*

One hundred per cent kill at 62°C. in sixty seconds, 59°C. in five minutes, and 56°C. in fifteen minutes. Temperatures maintained within less than ±0.5°C.

temperatures were attained during subsequent electrical treatment of inoculated juice.

The data are presented in figure 1. At sixty seconds the thermal death point was 62°C.; at five minutes, 59°C., and at fifteen minutes, 56°C. A temperature of 50°C. was the minimum lethal temperature in one minute, 48°C. in five minutes and 46°C. in fifteen minutes. "Lethal" is not used here to indicate 100 per cent kill, but merely to designate some killing action. At temperatures immediately below each minimum lethal point an acceleration of growth was observed. As the temperatures of the electrically treated solutions were within this range, it is possible that the action rendered the cells more susceptible to the electrical current.

Effect of amperage

A series of tests were conducted in which the amperage was progressively increased and the time of exposure was held constant. The data are given in figure 2.

In order to maintain non-lethal temperatures in the solutions, it was necessary to increase the diameter of the electrodes at the higher amperages; therefore, electrodes 0.95 cm.^{2} in area, 1.1 cm. in diameter were used at 0.1 to 0.23 ampere; 1.17 cm.^{2} in area at 0.23 to 0.3 ampere; and 4.9 cm.^{2} in area at 0.54 to 0.62 ampere. The approximate average inoculum was 1,500,000 cells per cubic centimeter, the minimum 135,000 per cubic centimeter, and the maximum 50,000,000 per cubic centimeter. In one instance 335,000,000 cells per cubic centimeter were used. This and other extreme values were not used in calculating averages. The larger inoculums did not presistently give higher percentage kills as was expected; however, comparative inoculums were not made in all the tests. The average indicated voltage at the electrodes was 22, the maximum 26, the minimum 15. The temperatures of the solutions varied from 30° to 48°C., the average being 44°C. In two instances at 0.23 amperes, the temperatures of the solution reached 50°C. in the last few seconds of the tests. Because the temperature could not be controlled satisfactorily, currents above 0.62 ampere were not used.

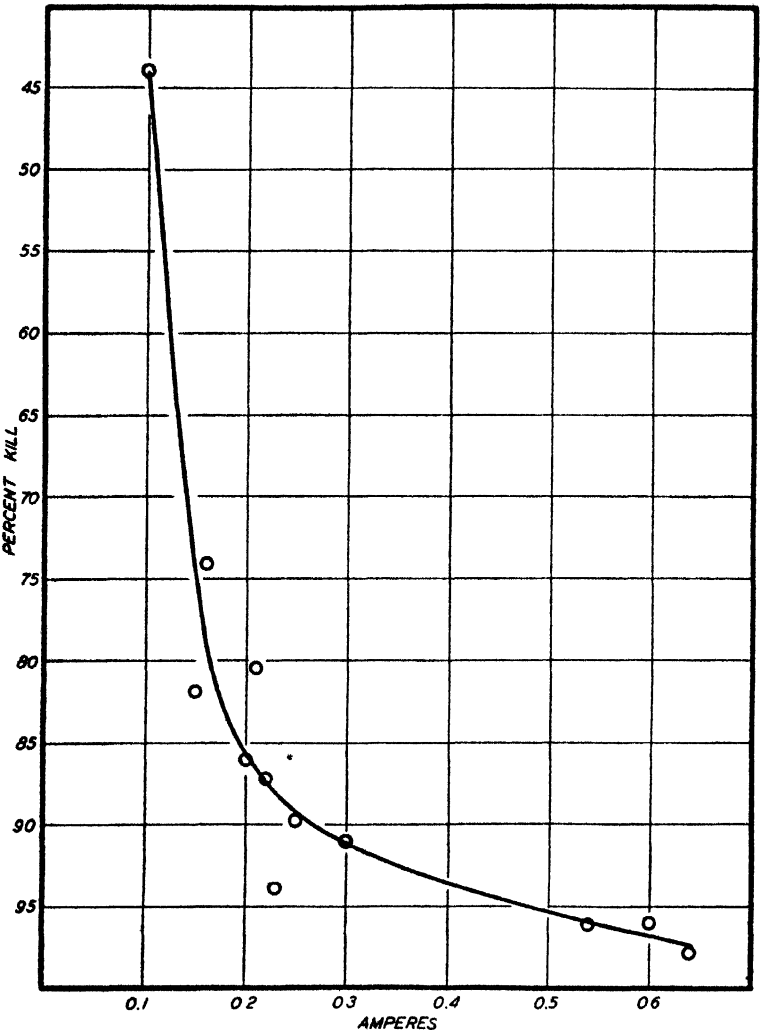

FIG. 2. RELATION OF AMPERAGE AND LETHAL EFFECT OF CURRENT

Medium, grape juice, 24 to 29 per cent solids, pH 3.1 to 3.5. Time of exposure, sixty seconds. Electrodes 0.95 cm.^{2} in area; 1.1 cm. diameter used in range 0.1 to 0.23 ampere. Electrodes 1.17 cm.^{2} used in range 0.23 to 0.3 ampere. Electrodes 4.9 cm.^{2} in area, 2.5 cm. in diameter, used in range 0.54 to 0.62 ampere. Average voltage, 22; average temperature, 44°C.

Figure 2 indicates that with increased amperage there is an increased percentage kill, and as the cells decrease in number from action of the current, their resistance to the killing action of the current increases.

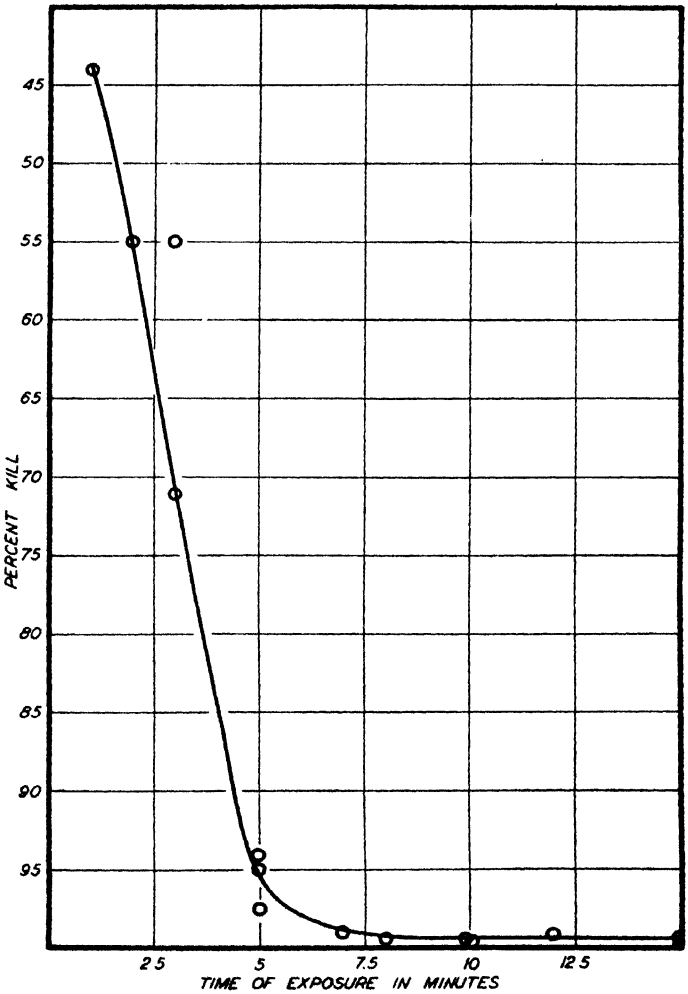

FIG. 3. EFFECT OF TIME OF EXPOSURE ON LETHAL ACTION OF CURRENT

Current, 0.1 ampere; time, one to fifteen minutes; medium, grape juice, 25 to 29 per cent solids; pH 3.1. Electrodes, 0.95 cm.^{2} in area; 1.1 cm. in diameter. Average voltage, 23; average temperature, 43°C.

Effect of time of exposure

In this series of tests, the amperage was held constant and the time of exposure progressively increased. The data are given in figure 3.

Electrodes 0.95 cm.^{2} in area (1.1 cm. in diameter) were used throughout the tests. The average voltage was 23, the minimum 20, and the maximum 28. The amperage was held constant at 0.1 ampere. The temperature ranged between 47° to 42°C., the average being 43°C. The inoculums averaged 370,000 cells per cubic centimeter; the minimum was 40,000 cells per cubic centimeter, and the maximum was 950,000 cells per cubic centimeter.

TABLE 1

Comparison of per cent kills by equal quantities of electricity applied at two different amperages

TEST	AMPERE	VOLTS	TEMPERATURE	TIME	CELLS PER CUBIC CENTIMETER		PER CENT KILL	QUANTITY OF ELECTRICITY	CURRENT DENSITY PER SQUARE CENTIMETER
					Before treatment	After treatment			
			°C.	*minutes*				*coulombs*	*amperes*
86 (3)	0.31	20	35	5	255,000	143,000	44	93	0.063
(4)	0.31	20	37	5	420,000	200,000	52.5	93	0.063
(5)	0.35	22	44	5	2,350,000	452,000	80.7	105	0.071
(8)	0.35	27	42	5	1,830,000	673,000	63.4	105	0.071
(6)	0.35	22	44	5	1,650,000	420,000	74.6	105	0.071
(7)*	0.10	20–22	43–44	15	408,000	462	99.7	90	0.14

* In (7) is given the averages of 4 tests at 0.1 ampere and fifteen minutes.

From the curve in figure 3, it is evident that higher per cent kills are obtained by longer periods of exposure, and that with the decrease in the number of surviving cells, an increase in resistance of the cells to the killing effect of the electrical current is manifested. In the interval from one to five minutes, the rate of disinfection is practically a straight line function and 96 per cent of the cells are destroyed. From five to fifteen minutes, the rate of disinfection decreases rapidly, remaining practically constant between ten and fifteen minutes. Unfortunately, longer periods of exposure with the present apparatus were impracticable, owing to the production of considerable gas from electrolysis, with the 60 cycle alternating current used.

It was thought that if the killing effect depended upon the quantity of electricity as indicated in figures 2 and 3, then it would be possible to increase the amperage and proportionately decrease the time of exposure, and still obtain, at equivalent energy input, per cent kills equal to those obtained with the original amperage and time of exposure. The data for such tests are reported in table 1. In these tests, 0.31 to 0.35 ampere and five minutes' exposure were substituted for 0.1 ampere and fifteen minutes' exposure. The data show that comparable results are not obtained by such a substitution, and indicate that the rate of destruction is dependent upon other factors in addition to the total input of electrical energy. In order to maintain the temperature within non-lethal ranges, electrodes 2.5 cm. in diameter, (4.9 $cm.^2$ in area) were used. This changed the current density significantly from 0.14 to 0.06 ampere per square centimeter, but not the quantities of electricity.

Comparison of current density and quantity of electricity

In figure 4 the per cent kills obtained in the previous tests are recorded in respect to the current density and time of exposure. Current density is defined in this instance as amperes per unit area of cross section of the conducting medium and is calculated by dividing the total amperage used by the area of the cross section.

Figure 4 indicates that the per cent kills are dependent upon current density as well as upon the total quantity of electricity. With a constant current density and progressively increasing time of exposure and quantity of electricity, an increasing per cent kill was obtained, as shown by the data for a current density of 0.14 ampere per square centimeter and one to fifteen minutes' exposure, columns 10 to 12. Using a constant time of exposure, and increasing the current density and quantity of electricity progressively, increasing kills were also obtained, as shown by comparison of data from 0.1 ampere, sixty seconds to 0.62 ampere, sixty seconds; columns 1 to 7. Likewise, the effect of quantity

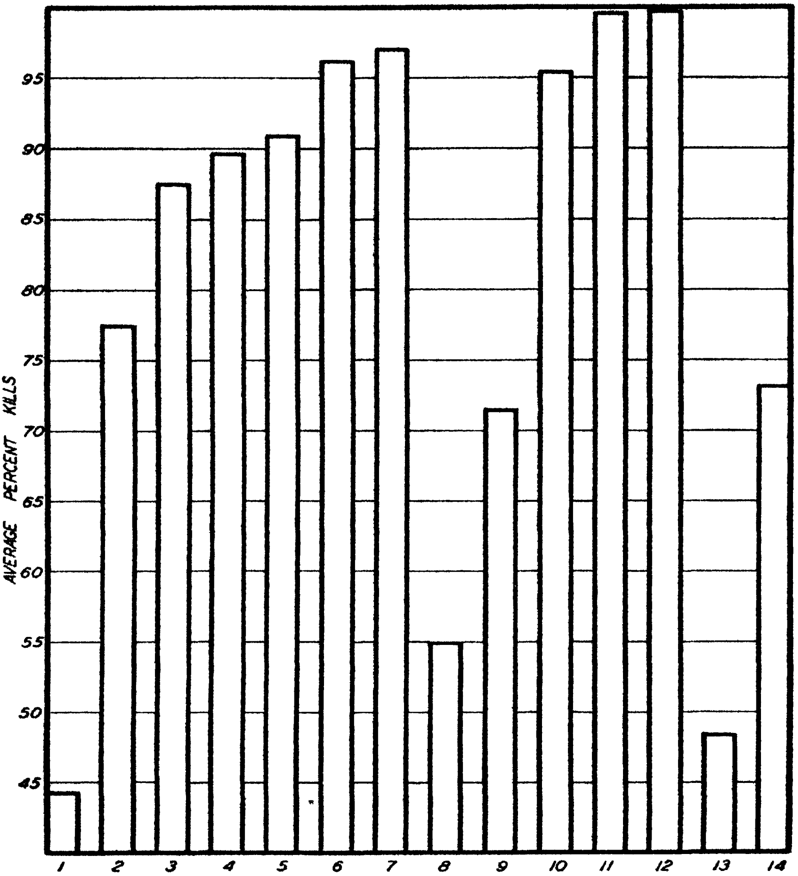

FIG. 4. GRAPHIC REPRESENTATION OF EFFECT OF CURRENT DENSITY AND QUANTITY OF ELECTRICITY ON LETHAL ACTION OF CURRENT

COLUMN NUMBER	EXPOSURE	AMPERE	CURRENT DENSITY	COULOMBS
1	60 seconds	0.1	0.10	6
2	60 seconds	0 17	0.17	10
3	60 seconds	0.22	0.22	13
4	60 seconds	0.25	0.22	15
5	60 seconds	0.3	0.25	18
6	60 seconds	0.54	0.11	32
7	60 seconds	0.62	0.126	37
8	2 minutes	0.1	0.10	12
9	3 minutes	0.1	0.10	18
10	5 minutes	0.1	0.14	30
11	10 minutes	0.1	0.14	60
12	15 minutes	0.10	0.14	90
13	5 minutes	0.3	0.06	90
14	5 minutes	0.35	0.07	105

of electricity is demonstrated by the tests at 0.54 and 0.62 ampere, sixty seconds with current densities of 0.11 and 0.12 ampere per square centimeter respectively, columns 6 and 7. The quantities of electricity in coulombs in these tests were approximately double that of the preceding tests, 0.25 and 0.3 ampere, 60 seconds, columns 4 and 5, but the current densities were only one half as high, 0.11 and 0.12 ampere per square centimeter compared to 0.22 and 0.2 amperes per square centimeter. Nevertheless, in spite of the lower current density, the kill was 5.5 per cent higher in the case of 0.54 and 0.62 ampere, sixty seconds, columns 6 and 7.

However, in tests with 0.3 and 0.35 ampere and five minutes' exposure, columns 13 and 14, the quantity of electricity in coulombs, 90 and 105 respectively, was 5 to 7 times greater than the quantity of electricity in coulombs of the test at 0.3 ampere, sixty seconds, column 5, but the current densities in the former tests were only one-fourth as great as the latter, and the observed kills were only 80 to 50 per cent as great as with 0.3 ampere, sixty seconds exposure. Likewise, in tests with 0.1 ampere, fifteen minutes' exposure, column 12, and 0.3 ampere, five minutes' exposure, column 13, the quantity of electricity in coulombs was equal, 90 coulombs each, but the current density was greater in the former than in the latter, namely, 0.14 and 0.061 ampere per square centimeter respectively, and the observed kills were 60 per cent greater with 0.1 ampere, fifteen minutes' exposure.

This seems to suggest that the bactericidal action of alternating current depends upon a definite quantity of electricity applied at, or above, a certain minimum current density in agreement with Thornton (1912) and Beattie and Lewis (1920).

Effect of electrolytic products on yeast

Although the electrolysis of solutions is much less with alternating than with direct current, it is not until a frequency of 1000 or more cycles is reached that this phenomenon becomes negligible, and with grape juice at 60 cycles considerable evolution of gases occurs. Qualitative tests showed these to be O_2 and H_2, as would be expected.

Free chlorine was tested for in the solutions with orthotoluidine, but in no case was it possible to obtain a positive indication.

No changes in the acidity and pH of the grape juice were detected after treatment with the electrical current in the series of tests reported above. This examination was not made for each test. Grape juice exposed to a current density of 0.11 ampere per square centimeter for thirty minutes at 42°C. was not altered in pH value.

The evolved gases were passed through yeast cell suspensions in order to determine whether or not they were toxic to the cells. In each case the viable counts of the suspensions approximated the control counts before treatment. Likewise it was determined that the electrical treatment did not alter the nutrient values nor impart a permanent toxicity to grape juice as follows. The growth of yeast was followed in untreated and electrically treated solutions previously electrolized at 0.1 ampere, thirty minutes at a current density of 0.105, temperature 42°C. After fifteen hours' observation the growth curves were found to be nearly identical.

To eliminate the possibility that certain compounds in grape juice might be toxic to the yeast cells while under electrical treatment, distilled water suspensions of yeast were treated with electricity in the same manner as those in grape juice. In these tests the voltage was 120, amperage 0.05 to 0.1, current density 0.05 to 0.105 ampere per square centimeter, and the time of exposure two and one-half minutes. The temperature was between 45° and 47°C. The observed kills were 85 to 87 per cent.

The flow of current through the solution was probably not uniform because of the presence of a meniscus, the layer of gas bubbles forming on the liquid surface, and the stratification effect in the absorption of heat by the cooling water; consequently an uneven temperature would exist throughout the medium. It was found that the highest temperature was approximately 3 to 4 mm. under the surface of the solution. In table 2 the temperatures obtained in various parts of the solution are given. The depth of the solution in these tests was approximately equal to the diameter of the electrodes.

Table 2 shows that non-lethal temperatures are maintained throughout the solution and that the lowest temperature is nearest the glass-liquid interface. This is probably due to the artificial cooling. Whether or not a lethal temperature is maintained in the immediate area surrounding individual cells has not yet been determined satisfactorily.

Although no toxic electrolytic products were detected by the methods employed, it is possible that free chlorine or other bactericidal substances are generated in the solutions only during the passage of electricity and are rapidly reduced, as is possible with free chlorine, upon opening the circuit so that it would be impossible to detect the presence of the substances later.

TABLE 2

Maximum temperatures in different parts of the solution

DIAMETER OF ELECTRODE	AMPERE	VOLTAGE	CURRENT DENSITY	LOCATION OF THERMOCOUPLE IN ELECTRICAL CHAMBER	TEMPERATURE
cm			*amp/cm.*^{2}		*°C.*
1.1	0.2	20	0.11	Medial, depth 3.4 mm.	48.8
				Medial, depth 6.0 mm.	47.7
				Medial, depth 9–10 mm.	42.7
				Medial, depth 11 mm. (glass-liquid interface)	36.6
				Electrode-liquid interface, depth 5 mm.	43.3
2.5	0.55	35	0.11	Medial, depth 4–5 mm.	48.3
				Medial, depth 10–11 mm.	46.6
				Medial, depth 19–20 mm.	42.8
				Medial, depth 25 mm. (glass-liquid interface)	36.6
				Electrode-liquid interface, depth 10 mm.	43.0

SUMMARY AND CONCLUSION

1. Lethal temperatures were quantitatively determined for a strain of *Saccharomyces ellipsoideus* in grape juice for one, five and fifteen minutes.

2. By passage of alternating current through yeast cell suspensions in grape juice at non-lethal temperatures, 42°C., pronounced

killing effects were obtained, indicating that alternating current of 60 cycles exerted a lethal action independent of temperatures.

3. The lethal effect of the current varied with the current density and quantity of electricity used.

4. Electrically treated grape juices, after prolonged exposures of thirty minutes, were not toxic nor lacking in nutrient values for yeast. The gases evolved from electrolysis were not toxic to yeast cells.

5. It is possible that the killing effect exerted by alternating current on yeast cells is caused by the formation of temporary toxic substances like free chlorine, and that these are immediately reduced upon cessation of the current, and thus disappear. Such an action as this would directly follow the electrical conditions determined as necessary for killing the yeast cells.

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THE INFLUENCE OF INORGANIC SALTS ON THE MULTIPLICATION OF GONOCOCCUS[1]

C. PHILLIP MILLER, Jr., A. BAIRD HASTINGS and RUTH CASTLES

From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago

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A preceding paper (Miller and Hastings, 1930) described an agar medium on which the gonococcus can be cultivated although it consists of only meat infusion, peptone, glucose and a solution of the salts present in mammalian plasma. Failure of growth in the absence of this mixture of salts seemed to indicate that the inorganic environment played an important rôle in the multiplication of this organism and stimulated the present inquiry into the relative importance of the various ions concerned.

This topic has received but little attention in the literature. The meningococcus has fared better in this respect, e.g., Flexner (1907) made some observations on the influence of calcium and potassium salts on the growth and survival of that organism, and Murray (1929) in his monograph on the meningococcus devotes several paragraphs to a discussion of the rôle of various inorganic ions in its biology.

MATERIALS

The medium employed was an egg-white digest agar, the formula for which has already been reported (Miller and Castles, 1930). As substrates for the digest, gelatin, peptone and casein were also used. Each yielded a product which supported growth of the gonococcus, but no more satisfactorily than that from egg-white. Various forms of this protein were tried: the whites of strictly fresh eggs, powdered egg-white as marketed by the commercial chemical firms, and the frozen, raw egg white used by

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bakers and confectioners. This latter preparation was less expensive than fresh egg-white, and quite satisfactory, as it contained no preservative. For a number of months powdered egg white was used, because of its convenience and low cost, and was reported in our preliminary communication as being entirely satisfactory. Since the time of that publication, however, three lots (identified by the manufacturer's lot numbers) have been encountered, which, on digestion, failed to support growth. As no explanation was found for the unsuitability of these samples, the use of fresh egg-white was subsequently resumed.

Preparation of the digest

The digest was made by dissolving 500 grams of fresh or frozen egg-white (or 40 grams of powdered egg-white) in a liter of water and adjusting the reaction to pH 7.0 to 7.4. Four grams of a special, high-test trypsin[2] (rubbed into a paste) were added and the mixture put into a galvanized kettle, placed in an electric water bath. The mixture was stirred by means of a paddle rotated by a water motor. The temperature was kept at 48°C., which permitted digestion to occur but inhibited the growth of most organisms. This arrangement for constant stirring is an unessential detail which was added because it enabled us to handle large batches of digest. When small quantities were to be digested they were placed in Erlenmeyer flasks, plugged with cotton, and agitated by hand from time to time. By the end of ten to twelve hours a sample of the digest usually gave an amino acid titration (i.e., cubic centimeters of N/10 sodium hydroxide per 100 cc. of digest) of 130 to 180 by the Sørenson method. After acidifying with HCl to pH 5.0 the heat-coagulable proteins were removed by autoclaving and filtering through paper. If part of the precipitate was so finely divided that it passed the paper filter, it was removed by filtration through a Berkefeld candle. Digest sterilized by Berkefeld filtration proved to be quite satisfactory after weeks of storage in the refrigerator.

To facilitate pH control of the media, phenol red was added in the proportion of 6 cc. of 0.04 per cent solution per 100 cc. of digest. This concentration of phenol red exerts no bactericidal action on the gonococcus. The reaction of the digest was then adjusted to pH 7.2 to 7.4 by the addition of normal sodium hydroxide. The amount required was accurately measured (as was the acid or base in subsequent adjustments) in order that it could be taken into account in reckoning the concentration of inorganic ions in the medium. The pH was determined with an accuracy of ±0.1 by comparing samples of the adjusted digest with standard pH solutions in a comparator block.

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[2] Obtained from the Wilson Laboratories, Chicago.

A typical detailed example of the preparation of a given batch of medium will illustrate the steps involved. Variations in the amounts of salts and other constituents used will be apparent from the tables.

Preparation of the control medium

This has been designated "control" medium because it has been found to be satisfactory for the routine cultivation of the gonococcus. A liter of it was prepared as follows:

Two hundred fifty cubic centimeters of digest were diluted with 750 cc. of distilled water. In this were dissolved, with heating, 0.2 gram potassium chloride, 3.0 grams sodium chloride, 1.25 gram sodium bicarbonate, 10 grams glucose, and 20 grams agar. Eight cubic centimeters of a 1/15 molar disodium hydrogen phosphate solution and 2.0 cc. of a 1/15 molar sodium dihydrogen phosphate solution were added and the reaction adjusted to pH 7.2 to 7.4 with hydrochloric acid. The medium was then tubed, autoclaved and slanted.

EXPERIMENTAL

The inorganic salt solution previously found to be suitable for growth of the gonococcus when combined with ordinary meat infusion and glucose agar was used as the standard solution of reference. This will subsequently be referred to as the "standard solution." Its composition is given in table 1. It was early found that the salts already present in the digest were in such a concentration that it was necessary to halve the amount of standard solution in order to obtain maximum growth. Technically, the procedure was this: 1 part of digest, 1 part of distilled water, and 2 parts of standard solution (without calcium chloride and magnesium chloride), glucose and agar were mixed, and the reaction adjusted to pH 7.4. The calcium and magnesium solutions were then added and the medium was tubed and autoclaved. The slants were cooled in an atmosphere of carbon dioxide until the color of the agar indicated a pH of 7.2 to 7.4.

The purpose of the carbon dioxide was to maintain a hydrogen-ion concentration which would prevent the precipitation of calcium and magnesium salts, for at this point in the development of the work we considered it important to provide the media with a mixture of ions simulating the inorganic composition of mammalian plasma. It was subsequently found, however, that this precaution which had been carried over from our previous work (Miller and Hastings, 1930) was unnecessary when egg-white digest was incorporated into the medium, and the use of carbon dioxide was discontinued.

TABLE 1

Composition of standard solution

CATION	mM/LITER	ANION	mM/LITER
Na^+	145.4	Cl^-	123.6
K^+	6.0	HCO_3^-	30.0
Ca^{++}	1.5	$HPO_4^=$	0.8
Mg^{++}	0 5	$H_2PO_4^-$	0.2
pH	7.4	CO_2 tension	46 mm. Hg

Experiments were then set up to determine the importance of the other constituents of the standard solution. Small batches of media were prepared in each of which one of the salts was omitted. Sodium bicarbonate appeared to be the one indispensable salt in the standard solution.

However, on analysis, our digest of egg-white was found to contain the following inorganic constituents in the concentration given:

	millimols per liter
Sodium	105
Potassium	12.3
Calcium	1.6
Chloride	116

Therefore, since the digest itself supplied a considerable amount of inorganic salts, an attempt was made to reduce these to a minimum by dialysis of the egg-white.

Digest of dialyzed egg-white

Dialysis of egg-white was carried out as follows: The whites of a dozen eggs (or 25 grams of powdered egg-white) were dissolved in 0.5 liter of 0.9 per cent sodium chloride and the solution acidified with hydrochloric acid to about pH 5.0. During the addition of the acid the solution was shaken in order to keep the precipitate which formed, as finely divided as possible. The mixture was then placed in cellophane bags and dialyzed in the refrigerator against distilled water, which was changed twice a day, for a week or ten days. At the end of that time the contents of the bag were filtered through paper and digested with trypsin as described above.

An analysis of the digest of dialyzed egg-white gave the following results:

	millimols per liter
Sodium	3 7
Potassium	0 3
Calcium	1.0
Chloride	8 0
Phosphate	1 8

Since a considerable portion of these inorganic ions was contributed by trypsin, an effort was made to free the trypsin of inorganic salts by dialysis, but this effort was unsuccessful.

Dialysis of the digest was, of course, impracticable because constituents necessary for growth, presumably the amino-acids, were thereby eliminated. It was proved by experiment that the dialysate, when concentrated and mixed with glucose and agar, supported growth.

THE EFFECT OF VARYING THE CONCENTRATION OF THE INDIVIDUAL INORGANIC IONS

Small batches of media were prepared whose inorganic composition with respect to certain ions was accurately known and their ability to support growth of the gonococcus tested by inoculating slants with viable cultures and examining them for growth at the end of twelve and twenty-four hours. If growth had occurred, a transplant was made from that slant to another of the same composition, in order to obviate the possibility that traces of some constituent essential for growth had been carried over in the inoculum. The results of these experiments are given in the subsequent sections. Final concentrations of inorganic ions are expressed in millimols per liter of agar gel. In their computation, account was taken of those supplied by the digest, those added directly to the medium and those added for purposes of adjustment of reaction.

Sodium chloride

Table 2 shows the effect of varying the content of sodium chloride from 892 to 5 millimols per liter. Sodium bicarbonate was present in a concentration of 15 millimols per liter. Other salts were present only to the extent to which they were contributed by the digest of dialyzed egg-white. Growth was good on the media whose concentrations of sodium chloride were 225 and 113 millimols per liter respectively. Outside these limits growth was poor and indeed absent when the concentration was 892 millimols per liter.

Table 3 contains the results of similar experiments, which differ from those of table 2 in that undialyzed egg-white was used in preparing the digest used. This table shows that growth was good when the sodium chloride concentration was 263 and 152 millimols per liter. Above and below these concentrations no growth occurred, or was poorly supported. The sum of important inorganic ions is given in the next to the last line of each table. Although these figures do not represent accurately the total osmolar concentration of the media, they may be regarded as proportional to it. The average osmolar concentration of normal plasma is approximately 300 millimols per liter. It is known that osmolar concentrations widely different from this figure will not permit the survival of mammalian cells. These experiments suggest that the osmolar concentration of the media must be

TABLE 2

Variations in the concentration of sodium chloride

Composition of media: Digest of dialyzed egg white, 25 per cent; agar, 2 per cent; glucose, 1 per cent. Initial pH, 7.2 to 7.4. Inorganic constituents as given in table. Concentrations expressed in millimols per liter.

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 4	EXPERIMENT 5	EXPERIMENT 6	EXPERIMENT 7	EXPERIMENT 8	EXPERIMENT 9
Na	915	470	248	136	81	53	39	32	28.5
K	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Ca	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Cl	892	447	225	113	58	30	16	9	5.5
HCO$_3$	15	15	15	15	15	15	15	15	15
PO$_4$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Osmolar concentration	1,823	933	489	265	155	99	71	57	50
Growth	0	+	+++	+++	++	+	+	+	+

TABLE 3

Variations in the concentration of sodium chloride

Composition of midia: Digest (undialyzed), 25 per cent; agar, 2 per cent; glucose, 1 per cent. Initial pH = 7.2 to 7.4. Inorganic constituents as given in table. Concentrations expressed in millimols per liter.

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 4	EXPERIMENT 5	EXPERIMENT 6	EXPERIMENT 7	EXPERIMENT 8
Na	943	498	278	164	70.2	66.4	55.9	542
K	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2
Ca	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
Cl	831	486	263	152	68.3	54.5	44	42.3
HCO$_3$	15	15	15	15	15	15	15	15
Osmolar concentration	1,794	1,004	561	336	158	140	120	116
Growth	0	0	+++	+++	+	0	0	0

restricted to similar limits in order to support the growth of gonococci. That failure to support growth in the high and low

TABLE 4

Variations in the concentration of potassium chloride

Composition of media: Digest of dialyzed egg white, 25 per cent; agar, 2 per cent; glucose, 1 per cent. Initial pH 7.2 to 7.4. Inorganic constituents as given in table. Concentrations expressed in millimols per liter.

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 4	EXPERIMENT 5	EXPERIMENT 6	EXPERIMENT 7	EXPERIMENT 8
Na	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
K	775.8	399.7	212.7	119.0	72.0	48.0	36.2	30.5
Ca	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Cl	753	377	190	96	49	25	13.5	7.8
HCO_3	16	16	16	16	16	16	16	16
PO_4	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Osmolar concentration	1,547	794	420	233	139	91	67	56
Growth	0	0	+++	+++	+++	++	+	+

TABLE 5

Variations in the concentration of potassium chloride

Composition of media: Digest (undialyzed), 25 per cent; agar, 2 per cent; glucose, 1 per cent. Initial pH = 7.2 to 7.4. Inorganic constituents as given in table. Concentrations are expressed in millimols per liter.

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 4	EXPERIMENT 5	EXPERIMENT 6	EXPERIMENT 7	EXPERIMENT 8
Na	52.5	52.5	52.5	52.5	52.5	52.5	52.5	52.5
K	755	380	192	98	27.7	10	5.6	4.9
Ca	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
Cl	791	417	229	135	64.1	46.4	42	41.3
HCO_3	15	15	15	15	15	15	15	15
Osmolar concentration	1,615	865	489	300	160	124	115	114
Growth	0	0	+++	+++	++	+	0	0

concentrations of sodium chloride is not due to the sodium ion *per se* is shown by tables 4 and 5.

Potassium chloride

In table 4 are shown results of a series of experiments in which only potassium salts were used in preparing the media. The potassium chloride concentration varied from 753 to 7.8 millimols per liter. The bicarbonate concentration was constant at 16 millimols per liter, and other ions were maintained at the minimum determined by their concentration in the digest of dialyzed egg-white. Growth was good on media whose potassium chloride concentrations were between 190 and 49 millimols respectively. Growth was poor or absent above and below these limits.

A similar experiment, in which digest of undialyzed, instead of dialyzed egg white was used, led to essentially the same results (see table 5). Good growth occurred in potassium chloride concentrations of 192 and 98 millimols per liter. Growth was poor or absent outside these concentrations. The limits of osmolar concentrations within which growth was well supported were 489 and 124. These experiments, taken in conjunction with the experiments of tables 2 and 3, demonstrate that potassium and sodium ions are equally suitable; that neither is itself toxic by virtue of its chemical individuality, and that neither is itself essential, providing the osmolar concentration of the inorganic constituents is within normal biological limits.

Calcium

The importance of calcium for the normal activity of all mammalian cells led to an attempt to determine whether or not it was essential for the gonococcus, and whether growth would occur when its concentration was high. In the media prepared from digest of dialyzed egg-white and to which no calcium salts were added excellent growth was obtained when the osmolar concentration was within normal limits. These media contained less than 0.25 millimols of calcium per liter. It proved to be impossible to remove the calcium completely from egg-white by dialysis. Media were therefore prepared which contained sodium oxalate in concentrations of 0.4 to 13 millimols per liter (table 6). Such concentrations are far in excess of the amount required to precipitate calcium from solution. Nevertheless these media supported growth even on repeated subcultivation, thereby demonstrating that oxalate ions are not in themselves toxic for the gonococcus and that calcium ions are not essential for its growth. Similar experiments, in which sodium citrate was substituted for oxalate, yielded the same results (table 6). Evidence that calcium is toxic in high concentrations is shown in table 7. When the calcium concentration was increased to 30 millimols per liter

TABLE 6

Effect of reducing calcium ion concentration

Composition of media: Digest (undialyzed), 25 per cent; agar, 2 per cent; glucose, 1 per cent. Concentrations expressed in millimols per liter.

	CONTROL*	INCREASING OXALATE				INCREASING CITRATE			
Na	98 5	98 2	98 2	98.2	98.2	100.3	108 7	129.2	140 2
K	3.1	7.4	8.7	19 7	32 0	6.1	6.1	6.1	6.1
Ca	0.25	0.4	0.4	0.4	0 4	0.4	0.4	0.4	0.4
Cl	87 6	83.1	83 1	83.1	83 1	83 1	83.1	83 1	83 1
HCO_3	15	15	15	15	15	15	15	15	15
Citrate						0 7	3 5	7.0	14 0
Oxalate		0.4	1.3	6 8	13				
Growth	+++	+++	+++	+++	++	+++	++	++	+++

* Dialyzed digest was used in this experiment and no calcium was added.

TABLE 7

Effect of increasing calcium concentrations

Composition of media: Digest (undialyzed), 25 per cent; agar, 2 per cent; glucose, 1 per cent. Initial pH, 7.4. Concentration expressed in millimols per liter.

Na	145	145	145	235	235	235
K	4.2	4.2	4 2	4.2	4.2	4.2
Ca	5.5	15 5	30.5	5.5	15.5	30 5
Cl	140	160	190	110	130	160
HCO_3	15	15	15	15	15	15
Citrate				60	60	60
Growth	++	++	+	++	++	+++

growth was inhibited, but when citrate was present in sufficient quantity to bind the calcium ions, good growth occurred.

Magnesium

Comparable experiments with magnesium seemed to show that, whereas 30 millimols of magnesium were unfavorable to growth, the presence of citrate did not diminish this toxic effect. Media prepared from digest of dialyzed egg-white to which magnesium was not added supported growth as well as media in which magnesium was present in a concentration of 1 millimol per liter.

TABLE 8

Effect of pH and of buffering on growth

Composition of media: Digest (undialyzed), 25 per cent; agar, 2 per cent; glucose, 1 per cent; salts (other than phosphate) as in control media.

A. Effect of pH in poorly buffered media (phosphate = 2.5 mM per liter)

Initial pH	5.5	6.2	6.8	7.2	7.6	8.0	8.4
Final pH	5.5	5.6	5.6	5.6	5.6	5.4	8.4
Growth	0	+	+	+++	+++	++	0

B. Effect of pH in strongly buffered media (phosphate = 32 mM per liter)

Initial pH	5.5	6.0	6.5	7.0	7.5	8.0	8.5*
Final pH	5.5	6.0	6.5	7.0	7.5	8.0	8.5
Growth	0	0	+	+++	+++	+	0

* Borate buffers were used instead of phosphate to obtain pH 8.5.

Hydrogen ion concentration

That the pH of the environment in which the organisms were cultivated is important for their continued growth is shown in table 8, A. Here the initial pH varied from 5.0 to 8.4. No growth occurred at a reaction below pH 6.2. Growth was good from 7.2 to 8.0 In these media the buffering was effected solely by 15 millimols of bicarbonate per liter and the amino acids present in the digest. The final pH was 5.4 to 5.6 in all tubes in which growth occurred, irrespective of the initial reaction.

In experiments in which the media were so strongly buffered with phosphate that the final pH was but slightly lower than the initial pH, good growth was obtained from pH 7.0 to 7.5, with sparse growth at 6.5 and 8.0 (table 8, B). This suggests that the pH limits within which multiplication of the gonococcus occurs corresponds to those of most biological organisms.

Bicarbonate

In experiment I it was noted that the omission of sodium bicarbonate from the medium considerably reduced its growth-supporting property. An experiment illustrating this point is represented by table 9. It will be seen that abundant growth occurred in media containing 15 millimols of bicarbonate per liter; but poor growth or none in those from which that ion was absent. It may be noted that the osmolar concentrations fell below the optimum and probably accentuated the deleterious effect of bicarbonate deprivation.

TABLE 9

Effect of removing bicarbonate

Composition of media: Digest of dialyzed egg white, 25 per cent; agar, 2 per cent; glucose, 1 per cent. Initial pH = 7.2 to 7.4. Concentrations expressed in millimols per liter.

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 4	EXPERIMENT 5
Na	73.2	58.2	58.2	98.5	83.5
K	3.1	3.1	3.1	6.1	3.1
Ca	1.0	0.3	1.0	0.4	0.4
Cl	64.6	61.8	64.1	87.6	84.6
HCO_3	15	0	0	15	0
Osmolar concentration	158	125	128	208	171
Growth	+++	+	+	+++	0

In another experiment (see table 10) the bicarbonate concentration was varied from 0 to 100 millimols per liter. The initial pH was brought to approximately 7.4 by adjusting with CO_2. Growth was good between 5.0 and 50.0 millimols of bicarbonate per liter, but above and below those concentrations growth was poor.

It is not believed that the apparent need for bicarbonate ions is due to any special virtue of the ion itself, but rather to its influence in maintaining within normal biological limits the hydrogen ion concentration of the bacterial cell and its environment. Absence of bicarbonate from the surrounding medium must permit diffusion of bicarbonate ions from the cell to the medium. In the event that CO_2 production continues at a normal rate, the lowered bicarbonate concentration within the cell would result in a lowered pH. This in itself might account for the failure of media without bicarbonate to support growth.

TABLE 10

Variation in concentration of sodium bicarbonate

Composition of media: Digest of dialyzed egg white, 20 per cent; agar, 2 per cent; glucose, 1 per cent. Initial pH = 7.4. Concentrations expressed in millimols per liter.

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 4	EXPERIMENT 5	EXPERIMENT 6
Na	150.7	150.7	150.7	150.7	150.7	150.7
K	0.06	0.06	0.06	0.06	0.06	0.06
Ca	0.02	0.02	0.02	0.02	0.02	0.02
Cl	151.6	146.6	141.6	121.6	101.6	57.6
HCO_3	0	5.0	10.0	30	50	100
PO_4	0.3	0.3	0.3	0.3	0.3	0.3
Growth	+	+++	+++	+++	+++	+

This is further substantiated by the fact that growth was well supported in a medium which was adequately buffered with phosphates instead of bicarbonate.

Two factors are probably concerned in the deleterious effect of high bicarbonate concentration. (1) The high CO_2 tension necessary for the maintenance of normal pH may be unfavorable to the growth of the organisms. (2) The high bicarbonate concentration of the environment may by diffusion into the cells bring about a sufficiently high concentration of this constituent to raise the pH within the cell above its physiological optimum.

That the lowered calcium ion concentration occurring in the media with large amounts of bicarbonate can not account for the inhibition of growth is evident from the section on calcium.

Other anions

That chloride is unessential for growth has been shown by the fact that cultures multiplied well in media in which salts of nitrate or sulfate had been substituted for chlorides.

It is true that these media were not chloride-free; but since its concentration did not exceed 3 millimols per liter, it is improbable that the chloride ion can be regarded as a constituent essential for growth. Of interest is the observation that nitrate concentrations of 100 millimols per liter and sulphate concentrations of 120 millimols per liter had no untoward effects. It may also be mentioned that good growth occurred in the presence of 30 millimols of phosphate per liter.

DISCUSSION

It is well known that animal cells are quite sensitive to changes in the concentration of the inorganic salts and in the osmotic pressure of the fluid surrounding them. For example, Dale (1913) has demonstrated that the response of the guinea pig uterus to drugs is markedly increased when the osmolar concentration of the surrounding fluid is decreased from 308 to 290 millimols per liter.[3] Plant cells, on the other hand, are more resistant to such changes in their environment. Many bacteria resemble vegetable cells in this respect. Thus, for example, *B. coli* will withstand such extremes as pure water and 9 per cent glycerol (Winslow and Falk, 1923, and Winslow and Holland, 1919). According to A. Fischer (1900) *B. subtilis* will grow in liquid media containing 9 per cent (1540 millimolar) sodium chloride, 5 per cent (936 millimolar) ammonium chloride, 11 per cent (1480 millimolar) potassium chloride or 10 per cent (1100 millimolar) potassium nitrate. Additional instances of analogous findings are cited in Falk's exhaustive review (1923) of the literature on this subject.

The gonococcus, according to our observations, is considerably more sensitive than the microörganisms just mentioned, to changes in the osmolar concentration of its environment. On the other hand, it is much less sensitive to such changes than are animal cells, and resembles the meningococcus, growth of which is reported by Gordon, Hine and Flach (1916) to diminish when the concentration of sodium chloride exceeds 1 per cent (171 millimolar) and to be inhibited at 4 (684 millimolar) per cent. Murray and Ayrton (1924) found that in the absence of added salts growth failed on their media or was at best poor. It is recognized, however, that the sole criterion of sensitiveness in their experiments, as in ours, has been ability to multiply. Had other biological functions been studied, it is possible that greater sensitivity would have been demonstrated. It is of interest that the mean osmolar concentration at which optimum growth occurred in our experiments corresponds approximately to the osmolar concentration of the body fluids of man, the natural host of the gonococcus.

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[3] For an adequate discussion of this point see Chapter VII in Bayliss, William M., Principles of General Physiology, 4th edition, London, 1924.

Another point of contrast between the gonococcus and cells of mammalian origin is the fact that the former apparently grew equally well when potassium salts were interchanged for sodium and when the calcium and magnesium salts were practically eliminated. That the organisms did not grow where calcium and magnesium were present in high concentration, however, demonstrates that the composition of the inorganic environment is not without its effect.

The toxic action of bivalent ions to microörganisms is well established. That the toxic action of calcium for the gonococcus is due to the calcium ions has been demonstrated in our experiments by the fact that when citrate is added, good growth is obtained in the presence of high calcium concentration. It is well known that citrate combines with calcium ions to form a soluble but slightly ionized calcium citrate.

With respect to the concentration of hydrogen ions, the limits within which multiplication occurred were definite but fairly wide. Although it seems from our experiments that bicarbonate ions are essential to growth, it is believed that the necessity for their presence rests in their providing the medium with a sufficiently high buffering capacity to keep the reaction within the limits necessary for life. The other inorganic anions studied were found to play no demonstrable rôle in promoting or preventing growth.

It may be concluded from our experiments, therefore, that in so far as multiplication may be taken as a criterion of biological activity, the gonococcus is influenced by its inorganic environment, but that the limits within which variations in concentrations affect growth are relatively broad.

SUMMARY

1. A study has been made of the rôle of certain inorganic salts in the multiplication of the gonococcus on artificial media.
2. Sodium and potassium were found to be interchangeable and non-toxic at high concentration.
3. Calcium and magnesium were unessential but inhibited growth at concentrations greater than 30 millimols per liter.
4. Citrate and oxalate nullified the toxic action of calcium but not of magnesium.
5. Growth occurred between pH 6.0 and 8.2 with optimum growth at pH 7.0 to 7.6.
6. Unless the media was well buffered either with bicarbonate or phosphate, growth did not occur.
7. Chloride was replaceable by nitrate or sulfate without impairing growth.
8. Below osmolar salt concentrations of 150 and above 550 millimols per liter, growth was inhibited.

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A NOTE ON REACTION CHANGES DURING STERILIZATION OF VEGETABLE EXTRACT CULTURE MEDIA

L. S. McCLUNG

Department of Bacteriology, The University of Texas, Austin

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In experiments with the thermophilic anaerobic group of organisms certain vegetable extract media have been found to be valuable. In various experiments using this type of medium considerable difficulty has been encountered with respect to reaction changes during sterilization in the autoclave. Sufficient lowering of pH was observed with thirty minutes autoclaving at 15 pounds pressure to change the color of media to which brom-thymol-blue had been added from deep blue to green or from grass green to yellow. Permanent disappearance of the color was also observed in certain vegetable media; namely, carrot and tomato.

Since exact information about the reaction changes occurring in media of this type as a result of sterilization was desirable, electrometric determinations have been made on certain vegetable media. These included pea, carrot and tomato.[1] Liver infusion medium was also included since it is a very common and valuable medium for the cultivation of thermophilic anaerobes. Plain beef-extract peptone broth was included in this series of determinations for comparison.

The medium being tested was tubed in large test tubes in about 40-ml. quantities. The reaction was determined by means of the hydrogen electrode, after which the tubes were marked, sterilized for the required time, and the determination of the reaction repeated. The vegetable media were also buffered with M/15 KH_2PO_4 and K_2HPO_4 and tested as above. The initial hydrogen

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[1] A 10 per cent solution of Gerber's Strained Vegetable in distilled water.

TABLE 1

MEDIUM	UNBUFFERED			BUFFERED		
	Initial pH	pH after autoclaving	Difference	Initial pH	pH after autoclaving	Difference
Carrot	5.4	4.9	−0.5	6.6	6.5	−0.1
	6.9	5.3	−1.6	6.8	6.7	−0.1
	8.8	5.7	−3.1	11.1	10.3	−0.8
	10.9	6.9	−4.0	12.2	11.9	−0.3
	11.6	10.3	−1.3	12.6	12.6	0
Tomato	4.3	4.3	0	6.5	6.4	−0.1
	7.4	5.8	−1.6	8.7	6.7	−2.0
	10.0	6.2	−3.8	11.6	10.8	−0.8
	11.1	7.1	−4.0	12.3	12.2	−0.1
	12.0	11.5	−0.5	12.8	12.7	−0.1
Pea	6.4	6.1	−0.3	6.5	6.5	0
	7.5	7.2	−0.3	7.1	7.0	−0.1
	8.0	7.5	−0.5	8.3	7.9	−0.4
	11.3	10.9	−0.4	11.3	11.1	−0.2
	12.0	11.7	−0.3	12.3	12.2	−0.1
Liver broth	6.4	6.4	0	6.1	5.9	−0.2
	6.8	6.6	−0.2	6.5	6.5	0
	9.0	8.1	−0.9	7.4	7.3	−0.1
	11.1	8.9	−2.2	10.9	8.7	−2.2
	12.1	11.5	−0.6	11.9	11.4	−0.5
Liver broth with dried liver				6.4	6.3	−0.1
				6.7	6.5	−0.2
				8.9	7.7	−1.2
				11.0	8.6	−2.4
				11.9	10.5	−1.4
Beef extract peptone broth	6.0	6.1	+0.1			
	7.0	6.9	−0.1			
	7.4	7.4	0			
	11.5	11.0	−0.5			
	12.3	12.2	−0.1			

ion concentration of the media ranged from pH 4 to 6 (unadjusted) to pH 12. It is realized that media with a pH of 12 will rarely or never be used in cultural work. These tubes were included in order to determine the extent of the change in reaction in media of high initial alkalinity. There appeared to be no significant difference in the changes in reaction occurring in hard and soft glass tubes.

Each figure in table 1 represents an average of at least three, and in the alkaline range of five, tubes, no one of which varied greatly from the average.

An examination of the results given in the table shows that in some cases the reaction change in vegetable media as a result of sterilization is quite marked. The most significant change, in the vegetables studied, occurs in the alkaline solutions of carrot and tomato. Marked change is also noted in liver infusion broth, both with and without the addition of ground liver. This reaction change cannot, in all cases, be controlled by the addition of buffer salts. Nor has it been found satisfactory to adjust the media to a reaction different from that which is to be used and depend upon sterilization to bring about the desired initial reaction. Adjustment of the reaction with sterile acid and alkali solutions after sterilization has been found to be the most satisfactory method for obtaining a desired initial reaction.

PASTEURIZATION OF MILK ARTIFICIALLY INFECTED WITH TWO STRAINS OF BRUCELLA SUIS

S. E. PARK,[1] ROBERT GRAHAM, M. J. PRUCHA AND J. M. BRANNON

University of Illinois

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[*Foreword:* Undulant fever as a public health problem is unique in many ways. It has been recognized as such only recently. That the principal source of the disease is in domestic stock no well informed observer doubts. There is no uniformity of opinion concerning the relative importance of channels through which the infection may reach man from animals. No student of the subject denies that infected milk may result in spreading undulant fever among humans.

A recent analysis of 155 cases of sickness that occurred during 1929 and 1930 in Illinois and which were clinically and serologically diagnosed as undulant fever cast a very strong suspicion on raw milk supplies as the agent of transmission in a significant percentage of the total incidence. Observers elsewhere have found evidence that infected milk may be an important means of transmitting the disease.

Furthermore, undulant fever prevalence may be on the up curve, potentially at least. If nothing is done to control the disease a great endemic wave of this ailment among men in the not far distant future is a catastrophe which is well within the realm of the possible. On the other hand a relatively small amount of judicious energy spent now in research and control may offset that possibility.

For these reasons it seems of the greatest importance to bring to light all possible knowledge about the cause of undulant fever and means of controlling the spread of it. The accompanying report is a contribution to an important phase of this knowledge. Some controversy about the efficacy of pasteurization in destroying the causative organisms of undulant fever has arisen. Doubts created by this controversy will survive until the matter is settled by indisputable scientific experimentation. This report might be accepted as closing the chapter on one phase of the necessary experimentation.—ANDY HALL, Director of Public Health, Chairman, State Undulant Fever Committee.]

Results of investigations made in recent years show that (*a*) cattle may be spontaneously infected with Brucella strains of the porcine type (Huddleson (1929)), (*b*) cattle may be artificially infected by intravenous injection of porcine cultures (Cotton (1922), Schroeder and Cotton (1925), Carpenter (1927), Graham, Boughton and Tunnicliff (1930)), (*c*) porcine strains may become established in the mammary glands of cows and may be eliminated in the milk (Smith (1929)), Hasseltine (1929), Carpenter and King (1928), Graham (1929)), and (*d*) *Brucella suis* may be isolated from the blood of undulant fever patients (Huddleson (1929), Evans (1927)). Although spontaneous cases of undulant fever have occurred when milk or milk products, so far as could be determined, were not a factor, contaminated milk and dairy products, *via* udder infection, must be considered a possible source of infection in brucelliasis (Carpenter and King (1928), Orr and Huddleson (1928), Murray (1929), Hardy (1929), Illinois Undulant Fever Committee (1929)).

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[1] Assistant Bacteriologist, State Department of Agriculture.

Since porcine Brucella types appear more pathogenic than bovine strains, the intermost susceptibility of cattle and man to porcine Brucella suggests the possible occurrence of a milk-borne, porcine Brucella infection. The possibility of cow's milk producing porcine brucelliasis in man emphasizes the importance of determining the thermal death time of different strains of *Brucella suis* in order to appraise the reliability of milk pasteurization standards in the prevention of porcine Brucella, milk-borne infections.[2]

HISTORICAL

Reported studies on the thermal death time of Brucella strains indicate that a temperature of 140°F. for a shorter time than that prescribed for pasteurization, i.e., thirty minutes, renders Brucella non-viable. McFadyean and Stockman (1909) reported that *Brucella abortus* in the moist state, when exposed to a temperature of 55°C. (131°F.), was destroyed in two hours, and was non-viable after an exposure of ten minutes in water at a temperature of 59 to 61°C. (138.2 to 141.8°F.). Zwick and Wedeman (1912) found that *Brucella abortus* was non-viable after being heated ten to fifteen minutes at 60°C. (140°F.), or five to ten minutes at 65°C. (149°F.). Fabyan (1913) reported that although heat resistance varied with different strains the average strain of *Brucella abortus* was non-viable after ten minutes' exposure to 59°C. (138.2°F.). Park (1927) found that composite cultures prepared from Brucella strains isolated from man, cattle, swine, and goats were killed when exposed for ten minutes to 140°F., seven and one-half minutes to 142°F., and five minutes to 145°F. These thermal death times were determined with artificially contaminated milk which contained 5,000,000,000 Brucella microörganisms per cubic centimeter. Boak and Carpenter (1928) found that an exposure of fifteen minutes to 140°F. destroyed the strains isolated from man, while a porcine strain was destroyed by an exposure of twenty minutes to 140°F. At 142°F. all strains were killed in from ten to fifteen minutes, while at 145°F. the same strains were killed in five minutes. In later experiments by the same authors (1931) temperatures of 142° and 145°F. for twenty and thirty minutes on the most virulent strains of *Brucella abortus* were found satisfactory for the pasteurization of milk. Traum (1930) found that melitensis and abortus strains were killed in fifteen minutes when exposed to 140 to 142°F., while a porcine strain was killed in twenty minutes at 140°F., or in fifteen minutes at 142°F. All three strains were destroyed in ten minutes when exposed to 145°F. Arnold and Gustafson (1930) reported that while caprine and bovine strains were destroyed in thirty minutes at a temperature ranging between 142 and 145°F., porcine strains were more resistant. In milk artificially contaminated with 25,000,000 Brucella microorganisms per cubic centimeter, one porcine strain proved viable after exposure to 146°F. for thirty-five minutes, but was destroyed in forty minutes. Hardy (1930) found strains of *Brucella abortus* non-viable after thirty minutes' exposure to temperatures of 144 to 145°F. Bartram (1931) reported that certain porcine strains may not be destroyed by heating to 140°F. for thirty minutes, to 142°F. for thirty minutes, or to 145°F. for fifteen minutes. One bovine strain was viable after thirty minutes at 142°F. Murray, McNutt, and Purwin (1932) found that 62 to 63°C. (143.6 to 145.4°F.) in a standard pasteurizer was sufficient to destroy porcine and bovine Brucella strains in three minutes when the pasteurizer was closed. The same degree of temperature applied to the pasteurizer with open lid did not destroy Brucella in the milk foam in thirty minutes. The published data indicate that porcine strains are more resistant than bovine or caprine varieties.

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[2] The Illinois Department of Public Health Proposed Milk Ordinance for Municipalities of Illinois designates the heating of milk for thirty minutes at 142 to 145°F. for pasteurization.

HEAT RESISTANCE OF TWO PORCINE STRAINS

1. *Heating of contaminated milk samples*

In the study of heat resistance of porcine Brucella, two strains, 2012 and 2872, in artificially contaminated, sterile whole and skim milk samples, were exposed for thirty minutes to temperatures ranging from 134 to 144°F. Strain 2012 was isolated from an aborted, porcine fetus (1922), while strain 2872 was isolated from a spontaneous, osteomyelitic lesion (James and Graham (1930)). Cultures of the two strains grown on agar slants, incubated twenty-four to forty-eight hours at 37°C. and suspended in sterile physiological salt solution, were used as the inoculum. The number of organisms per cubic centimeter was determined by hemocytometer and plate counts. The contaminated milk was heated in approximately 5 cc. amounts in cotton-stoppered tubes, and in 2 cc. amounts in hermetically sealed glass tubes. While it is apparent that Brucella strains might survive a longer period of time in the dried film that sometimes forms on the side of the cotton-stoppered tubes during heating, inasmuch as a comparable milk line may appear in pasteurization vats, this objection may not be of practical significance. However, duplicate contaminated milk samples were heated in sealed tubes for comparison.

The temperatures at which the tubes were heated were maintained in a double boiler and an electrically controlled, constant-temperature water bath. The temperatures in the double boiler bath were determined by a thermometer immersed in a cotton-stoppered tube of milk, placed in the boiler along with the artificially inoculated tubes. The temperature readings were checked by a second thermometer placed directly in the water. The inoculated tubes and the control tube, with the thermometer, were placed in the bath simultaneously, and the tubes were allowed to reach the specified temperature before beginning the heating period. For sealed tubes the heating period was considered as beginning at the time when the temperature of the water bath became constant after the tubes were added. The temperature did not vary more than one degree during heating. After heating, the tubes were immediately cooled in ice water. To check the accuracy of results obtained with double boiler water bath temperatures, samples of the artificially inoculated milk were heated in an automatic, electrically controlled, water bath. The effect of heat in the double boiler water bath on the two porcine Brucella strains appeared comparable to that obtained with electrically controlled heating.

2. Bacteriologic examination of heated contaminated milk samples

Bacteriologic examination of the artificially contaminated milk, subjected to different temperatures, consisted of plating, on media favoring the growth of Brucella, 0.2 cc. of milk sediment (skim milk) or cream (whole milk) from each sample, immediately after the tubes were cooled. In some cases duplicate cooled samples, or the remainder of the cultured samples, were incubated at 37°C. for four days before culturing. The sediment or cream of the heated samples was streaked on agar plates or inoculated into melted agar and poured into sterile petri dishes. Cultures were incubated for four days at 37°C. Colonies resembling Brucella were transferred from the plates to agar slants which were incubated at 37°C. for two to four days. If the growth, resulting from single colonies picked from the agar streak or poured plate, resembled Brucella culturally (H_2S production and negative carbohydrate reactions) it was identified serologically with Brucella immune and negative serums in dilutions of 1:50, 1:100 and 1:200.

3. Results of bacteriologic examination of heated contaminated milk samples

A. Samples heated in cotton-stoppered tubes

1. Effect of temperatures 140 to 144°F. on strains 2012 and 2872 (5000 Brucella organisms per cubic centimeter) in skim milk. Six samples of milk, inoculated with approximately 5000 Brucella organisms per cubic centimeter, of strain 2012, and 6 samples similarly inoculated with strain 2872 were heated for thirty minutes, 2 samples each at 140, 142, and 144°F. Plate cultures of the heated samples (unincubated) did not yield Brucella. The portions of the milk samples remaining after the plate cultures were made were incubated at 37°C. for two days. The incubated samples inoculated with strain 2012 were administered orally in amounts of 1 cc. to guinea pigs, while the incubated milk samples inoculated with strain 2872 were injected subcutaneously into guinea pigs in 1 cc. doses. The guinea pigs were necropsied at the end of twenty-seven days. No gross, pathologic lesions of brucelliasis were found, while direct cultures of the heart blood, liver, and spleen proved negative. The blood serum failed to agglutinate porcine Brucella antigen

2. Effect of temperature 144°F. on strains 2012 and 2872 (50,000, 1,000,000, and 10,000,000 Brucella organisms per cubic centimeter) in skim and whole milk. Twelve samples of skim milk and 12 of whole milk, in groups of 4, inoculated with strain 2012 and a similar number inoculated with strain 2872 were heated for thirty minutes at 144°F. The amount of inoculum varied so that samples contained 50,000, 1,000,000, and 10,000,000 Brucella organisms per cubic centimeter. Incubated and unincubated, heated, whole milk samples, containing approximately 10,000,000 organisms per cubic centimeter of strain 2012 and of strain 2872, yielded viable organisms. Incubated and unincubated, heated, whole milk samples containing fewer Brucella organisms per cubic centimeter of either strain 2012 or strain 2872 were negative. From incubated, heated, skim milk samples, containing 1,000,000 and 10,000,000 organisms per cubic centimeter of strain 2872, viable Brucella was isolated. However, incubated, heated, skim milk samples containing fewer Brucella of strain 2872 and those inoculated with strain 2012 did not yield viable Brucella. All unincubated, heated, skim milk samples failed to yield viable Brucella.

3. Effect of temperature 144°F. on strains 2012 and 2872 (100,000,000 Brucella organisms per cubic centimeter) in whole and skim milk. Six skim milk samples and 10 whole milk samples containing approximately 100,000,000 Brucella organisms per cubic centimeter of strain 2012 and a like number of samples, similarly inoculated with strain 2872, were heated thirty minutes at 144°F.[3] Viable Brucella was recovered from incubated and unincubated, heated, whole and skim milk samples inoculated with strains 2012 and 2872.

4. Effect of temperature 144°F. on strains 2012 and 2872 (500,000,000 Brucella organisms per cubic centimeter) in whole milk. Three whole milk samples containing approximately 500,000,000 organisms per cubic centimeter of strain 2012, and 3 samples similarly inoculated with strain 2872 were heated fifty minutes at 144°F.[4] Samples, plated immediately after cooling, yielded viable Brucella. No samples were incubated.

B. Samples heated in hermetically sealed glass tubes

1. Effect of temperature 144°F. on strains 2012 and 2872 (100,000,000 Brucella organisms per cubic centimeter) in whole milk. Whole milk samples, inoculated with strain 2012 and strain 2872, 100,000,000 Brucella organisms per cubic centimeter, were sealed in glass tubes and heated for thirty minutes at 144°F. Three tubes of milk inoculated with each strain were removed at two, five, seven, ten, fifteen, twenty, twenty-five and thirty minutes. These samples were cooled in ice water and cultured immediately. Samples containing strains 2012 and 2872 yielded viable Brucella after being heated two and five minutes but were negative after being heated seven minutes.

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[3] The remainder of this contaminated milk was heated in hermetically sealed glass tubes—see Results B 1.

[4] The remainder of this contaminated milk was heated in hermetically sealed glass tubes—see Results B 2.

2. Effect of temperatures 134, 140, 142, and 144°F. on strains 2012 and 2872 (500,000,000 Brucella organisms per cubic centimeter) in whole milk. Whole milk samples artificially contaminated with strain 2012 and with strain 2872, approximately 500,000,000 Brucella organisms per cubic centimeter, were sealed in glass tubes and heated at 134, 140, 142 and 144°F. for thirty minutes. Three samples containing each strain were removed at two, five, seven, ten, fifteen, twenty, twenty-five and thirty minutes, cooled in ice water, and cultured immediately. Viable Brucella was recovered from samples heated at 134°F. for thirty minutes. At 140°F. Brucella was viable for fifteen minutes but non-viable in twenty minutes. Brucella remained viable for ten minutes at 142°F. and five minutes at 144°F., but was non-viable in fifteen and seven minutes, respectively.

DISCUSSION

The data suggest a variance in the thermal death time of two porcine cultures, strain 2012 appearing less heat resistant than strain 2872. Since this was observed only once it is possibly not significant. Variation in thermal death time, however, was noted as the numbers of Brucella organisms placed in the milk varied. The time required to kill Brucella in contaminated milk, therefore, appears dependent upon the degree of contamination, or the number of organisms. This may account for the variation in the thermal death time obtained with the two porcine strains. The thermal death times of the two strains of porcine Brucella were much lower in sealed tubes than in cotton-stoppered tubes. Sealed tubes prevent a milk film from forming during heating. Organisms might survive longer in such a dried film than in the milk sample.

It is conceded that the massive inoculation used in some cases for the artificial infection of milk would seldom be encountered in freshly drawn milk. The number of organisms secreted from the udder appears variable, the greatest number being present in the milk soon after abortion. Brucella organisms appearing spontaneously in milk are greatly reduced or may completely disappear in three or four weeks. In some animals the udder infection persists for months or years. The Mediterranean Fever Commission (1887) reported a variation of the number of Brucella organisms secreted in goat's milk from none to 30,000 per cubic centimeter. Hasseltine (1929) stated that *Brucella abortus* is not present in large numbers in milk—50,000 per cubic centimeter being an exceptionally high number. Carpenter and King (1929) reported 20 to 500 Brucella organisms per cubic centimeter from market milk, while Hasley (1930) obtained an average of 2 organisms per cubic centimeter of certified milk (highest count being 8). Evans (1918) reported 145,000 Brucella organisms per cubic centimeter in the milk of an artificially infected cow, but it was observed that virulent organisms were not continually secreted. Since it is difficult to appraise the conditions in the udder which influence Brucella growth, the numbers encountered when the milk is plated are at best but an index range without reference to a definite degree of infection.

It appears that under certain conditions infected milk may become a medium for the growth and multiplication of Brucella. However, bacterial growth in such samples possibly retards Brucella multiplication and growth by decreasing the pH (below 5) (Carpenter and Boak (1928)).

SUMMARY

1. Two strains of *Brucella suis*, in hermetically sealed glass tubes of whole milk (500,000,000 organisms per cubic centimeter), were non-viable after twenty minutes at 140°F., after fifteen minutes at 142°F., and after seven minutes at 144°F.

2. The same strains proved more resistant to heat in cotton-stoppered tubes of milk. *Brucella suis* survived for thirty minutes at 144°F. in milk containing 10,000,000 to 500,000,000 organisms per cubic centimeter, but the same period of time at the same temperature destroyed *Brucella suis* in milk containing 5,000 to 1,000,000 organisms per cubic centimeter. Therefore, it appears that the thermal death time is influenced by the degree of contamination.

3. The data suggest that efficient pasteurization will prevent milk-borne porcine brucelliasis. However, final conclusions are withheld pending results of studies on commercial pasteurizers.

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TYPES OF BACTERIA ON BLOOD AND CHOCOLATE AGAR AND THE IMMEDIATE CAUSE OF THESE TYPES

EINAR LEIFSON

Department of Pathology and Bacteriology, Johns Hopkins University, Baltimore, Maryland[1]

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The action of certain bacteria on blood agar is so striking and constant a phenomenon that it has come to be of considerable importance in diagnosis. This is especially true in the case of the cocci. The effect on blood agar of bacteria other than the cocci has been described from time to time but no systematic study of a large number of types seems to have been made. Paulson and Brown (1931) have recently described colonies of colon bacilli of the alpha, beta, and gamma types.

The present paper deals with the effect on blood agar of some 260 strains of Gram-negative bacilli and 14 selected strains of various types of streptococci and pneumococci. The probable cause of the green coloration produced by some of these bacteria has been determined, and the various factors which influence the production of the green coloration have been investigated. A study has also been made of the effect on chocolate agar of both bacilli and cocci. It must be emphasized at this time that the types of bacterial colonies in blood agar about to be described are all deep colonies. Many deep colonies of the gamma (non-hemolytic) type may be considerably more hemolytic as surface colonies.

The appearance of different types of colonies of streptococci in blood agar has been described by a number of investigators and especially by Brown (1919) in whose paper will be found a

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review of the literature up to that time. The three distinct types of deep colonies of streptococci in blood agar are now well known, as are also the designations alpha, beta, and gamma given by Smith and Brown (1915). A fourth type, delta, was described by Bryant (1925), but the difference between this type as described by Bryant, and the alpha type seems to have been too slight to gain general recognition. Tunnicliff (1930) has found that some beta streptococci will give a green coloration on chocolate agar while others will not. From the literature referred to and the data which will be presented in this paper, we may now recognize two distinct kinds of bacteria which produce the alpha type of colony. The beta streptococci may also be divided into two types. A brief description of these types follows:

1. The gamma type produces no visible change of the corpuscles of a blood agar plate. None of the strains studied produced any visible effect on chocolate agar. This does not exclude the possibility that such strains exist, however. There are also a number of bacteria, both cocci and bacilli, which produce deep colonies of the gamma type but whose surface colonies are hemolytic. Staphylococci of this type are often found.

2. The beta type produces clear zones of hemolysis around the deep colonies in a blood agar plate. Occasionally bacteria are found which produce deep colonies having beta-like zones of partial hemolysis. These have been designated as alpha prime by Smith and Brown (1915). They are perhaps either to be considered as atypical beta types or atypical alpha. On chocolate agar some streptococci of the beta type produce a green coloration which according to McLeod and Gordon (1922) is due to the production of hydrogen peroxide. Others produce no visible effect. The former of these may be designated beta-H_2O_2 positive and the latter beta-H_2O_2 negative. More exhaustive investigation is needed to show whether the production of hydrogen peroxide by some streptococci of the beta type is sufficiently constant and clear-cut to be of classificatory significance.

3. The alpha type generally produces a green coloration around the deep colonies in a blood agar plate. In case of the bacilli of the alpha type the green coloration is very pronounced and the

accompanying hemolysis rather slight. Streptococci and pneumococci of the alpha type often produce a barely perceptible amount of green with considerable hemolysis. In many cases only microscopic examination of the colonies will determine their type. For a detailed description of the microscopic appearance of the alpha type the reader is referred to the monograph by Brown (1919). All of the strains of alpha streptococci and pneumococci studied produced a green coloration on chocolate agar. None of the alpha bacilli studied produced any green coloration on chocolate agar. Subsequent discussion will show that the two types of alpha bacteria, as determined by chocolate agar, differ fundamentally physiologically as well as morphologically in all cases which we have studied. The alpha bacilli produce a green coloration of blood agar by virtue of the hydrogen sulphide which they produce. The alpha cocci change hemoglobin to hematin and also produce hydrogen peroxide which reacts with hematin to give a green coloration. Rather than introduce a new name it

TABLE 1

Types of bacterial colonies in blood and on chocolate agar

TYPE	BLOOD AGAR	CHOCOLATE AGAR
Gamma	No visible effect	No visible effect
Alpha-H_2S positive	Large green zone with little if any hemolysis. Appearance due to the production of H_2S, and subsequent oxidation by air of the sulfhemoglobin formed	No visible effect
Alpha-H_2O_2 positive	Green zones with partial hemolysis. Appearance due to the formation of hematin, and H_2O_2 which react to form the green coloration	Green coloration. Due to interaction of hematin and H_2O_2
Beta-H_2O_2 negative	Large zones of complete hemolysis No green	No visible effect
Beta-H_2O_2 positive	Large zones of complete hemolysis. No green	Green coloration. Due to interaction of hematin and H_2O_2

was decided to refer to the one group of alpha bacteria as alpha-H_2S positive, and to the other as alpha-H_2O_2 positive. The term alpha as here used is purely descriptive of the appearance of the deep colonies in blood agar. In a report of this work given before the last meeting of the Society of American Bacteriologists the term delta was used for what we now call alpha-H_2S positive.

THE CAUSE OF THE GREEN COLORATION

The alpha-H_2O_2 positive type

The bacteria which produce this type of colony are often referred to as methemoglobin producers. This designation has come about perhaps from the work of Cole (1914) and Blake (1916) who showed that some green-producing streptococci and pneumococci generally produce methemoglobin when blood is added to a broth culture. Lyall (1914) found that some green-producing streptococci produce little if any methemoglobin while others produce considerable. Our own observations confirm those of Lyall. The green-producing bacilli which we have designated as alpha-H_2S positive do not seem to produce demonstrable amounts of methemoglobin. From these observations it is clear that there is no general correlation between the production of methemoglobin and a green coloration of blood agar. The suggestion is made that the term "methemoglobin producers" as referring to green producing cocci should be discontinued. McLeod and Gordon (1922) found a perfect correlation between the production of hydrogen peroxide and a green coloration of heated blood agar. These observations have been abundantly confirmed, especially by the exhaustive work of Avery and Neill (1924), Neill and Avery (1924, 1925), and Neill (1925). Although such oxidizing agents as sodium perborate will also produce a green coloration of heated blood agar, hydrogen peroxide is most likely responsible for the green coloration produced by bacteria in heated blood agar. The inference seems to have been made by some that hydrogen peroxide is responsible for the green coloration produced by bacteria in blood agar as well as in chocolate agar. Hydrogen peroxide, however, does not produce a green coloration of blood agar and cannot, therefore, alone be the cause of the green coloration. Hagan (1925) suggested that the simultaneous production of acid and of hydrogen peroxide might be the cause of the green coloration. The amount of acid however, in combination with hydrogen peroxide, which is necessary to produce a green coloration is far in excess of that generally produced by these bacteria in blood agar. This explanation is therefore not very probable. Ruediger (1906) found that certain hemolytic streptococci would produce a green coloration in blood agar if a fermentable carbohydrate (glucose) was added. He concluded that the green coloration was due to the action of some acid on the erythrocytes of the blood. No such acid is known, however, and this explanation is not very probable.

When blood is treated with acid, sodium nitrite, or heated to 70°C. or above, the hemoglobin is broken down and hematin is formed. Hematin will react with hydrogen peroxide to give a green coloration but hemoglobin will not. Blood which has been treated with potassium ferrocyanide does not react with hydrogen peroxide to give a green coloration. This may be taken to show that hydrogen peroxide does not react with methemoglobin to give a green coloration. Any bacteria, therefore, which produce hydrogen peroxide will give a green coloration on blood agar which has been heated or treated with chemicals in such a way that hematin is produced, but will not necessarily give a green coloration on whole blood agar or on methemoglobin agar. The exact mechanism of the production of hematin from hemoglobin is still somewhat obscure. For a detailed discussion of this subject the reader is referred to the work of Avery and Neill. According to these authors the hemoglobin is first changed to methemoglobin by means of oxidizing enzymes. The methemoglobin is further changed by enzymes to form hematin. From the work of Lyall (1914) and also from our own observations it seems that in some cases methemoglobin is not formed to an appreciable extent. This does not prove that methemoglobin is not always an intermediary product in the change of hemoglobin to hematin, but rather that in some cases it is too transitory to be detected. The change of hemoglobin to methemoglobin and hematin takes place only under aerobic conditions and consequently no green coloration is produced by the alpha cocci under anaerobic conditions.

The cause of the hemolytic zone around the colonies of alpha cocci is somewhat obscure. In some ways it would seem to be due to an agent distinct from that which causes the green coloration. Six strains of alpha streptococci and pneumococci, for example, produced a distinct hemolysis but no green on a synthetic blood agar the composition of which is given later (see fig. 4, plate 1). On the other hand, 5 of the 6 cocci when grown under strictly anaerobic conditions produced no hemolysis and no green, while one produced both hemolysis and a green coloration. Those alpha strains which are gamma under strictly anaerobic conditions become somewhat hemolytic when the oxygen tension is slightly increased, but no green coloration is apparent until the oxygen tension is increased still further. The beta type of hemolysis does not seem to be influenced by the oxygen tension. From these data it seems that the production of hemolysis and of a green coloration by alpha cocci are not independent phenomena, but that the two are caused by the same agent. Hemolysis may be a manifestation of a slight injury to the erythrocytes and may be brought about in a variety of ways. For example, if part of a blood plate is heated on a steam bath a wide zone of hemolysis appears between the zones of chocolate agar and of unchanged blood. Hemolysis may be brought about also by the action of acids, hydrogen peroxide, etc. From these data it seems most likely that hemolysis produced by the alpha type of cocci is due to very much the same substances which produce the green coloration, and is associated with a slight injury of the erythrocytes.

To summarize the course of the formation of the alpha-H_2O_2 positive type of colony: The oxyhemoglobin is first reduced to hemoglobin. In some way, as yet poorly understood, the hemoglobin is changed either first to methemoglobin and then to hematin and globin, or the hematin may be formed directly from the hemoglobin. The hematin reacts with the hydrogen peroxide formed by the bacteria to produce a green pigment. Both of these reactions take place only in the presence of air. Due, perhaps, to hydrogen peroxide or some other substance produced by the bacterial metabolism, some of the erythrocytes are slightly injured and a zone of partial hemolysis is formed around the colony.

The alpha-H_2S positive type

The alpha-H_2S positive type has been distinguished from the alpha-H_2O_2 positive type by its failure to produce a green coloration on chocolate agar, and by its large zone of green coloration on blood agar, with little or no hemolysis. All green-producing bacilli studied belong to the alpha-H_2S positive type. The alpha type of bacilli produces no hydrogen peroxide, and neither methemoglobin nor hematin from hemoglobin. From the study of some 260 strains of Gram-negative bacilli a perfect correlation was found between the formation of a green coloration on blood agar and the production of hydrogen sulphide. Investigation of the effect of hydrogen sulphide on blood showed that it reacts with hemoglobin to form purple sulfhemoglobin. Sulfhemoglobin has a strong absorption band at about $620\mu\mu$. This absorption band may be distinguished from that of methemoglobin by the addition of ammonium hydroxide which causes the methemoglobin band to disappear but leaves the sulfhemoglobin band intact. Carbon monoxide will cause a shift of the sulfhemoglobin band to about $610\mu\mu$ but has no effect on the methemoglobin band. Sulfhemoglobin is easily oxidized either by air or by oxidizing agents such as sodium perborate to form a green compound. Sulfhemoglobin is rather difficult to demonstrate in blood cultures of alpha bacilli unless substances such as cysteine or thiosulphates are present. Blood plates exposed aerobically to hydrogen sulphide turn green immediately and no sulfhemoglobin can be detected in the plates. Neither can any sulfhemoglobin be detected in the green zones around the colonies of alpha bacilli. The green compound does not seem to have a characteristic spectrum. One typical strain of an alpha colon bacillus grown on blood agar plates under anaerobic conditions for forty-eight hours produced no green coloration. Exposure of these plates to air in the refrigerator did not lead to the production of a green coloration, but exposure at room temperature did. Compounds such as cysteine and sodium thiosulphate greatly stimulate the production of a green coloration by alpha bacilli.

Hydrogen sulphide does not produce a green coloration of chocolate agar, nor does it react with hematin to give a green coloration. Neither do alpha bacilli, in our experience, ever produce a green coloration on chocolate agar.

The effect of hydrogen sulphide on blood has been known for some time. Harnach (1899) studied the effect of hydrogen sulphide on blood and noted the development of a green coloration in the presence of air. V. d. Bergh (1905) found that the blood of some of his enteritis patients showed a typical sulfhemoglobin spectrum. Addition of blood to bouillon cultures of the specific organisms gave, after twenty fours of incubation, a typical sulfhemoglobin spectrum. He concluded that the sulfhemoglobin in the blood of his patients was caused by hydrogen sulphide formed by the organisms in the intestine.

The data presented above seem to the writer to be adequate proof that the alpha type of colony, produced by the bacilli investigated, results from the interaction of hydrogen sulphide and hemoglobin to form sulfhemoglobin. The sulfhemoglobin thus formed is quickly oxidized to form a green compound. Any hemolysis formed around this type of colony is probably due to some metabolic product of the bacteria, perhaps hydrogen sulphide.

EFFECT OF THE PHYSICAL AND CHEMICAL ENVIRONMENT ON THE PRODUCTION OF THE GREEN COLORATION

The effect of the physical and chemical environment on a biological reaction does not seem to be fully appreciated. It is well known that chemical reactions are modified and at times completely changed by changing the environment. This is even more true in the case of biological reactions. Not only must we consider the effect of the environment upon the chemical reactants and resultants, but also the effect of the environment upon the living matter producing the chemicals. The environment is especially important in cases where it forms, or may form, one of the reactive agents. Such is the case with bacterial oxidations or reductions in which the atmospheric oxygen may play an important rôle. The formation of a green coloration by alpha bacteria in blood agar is partly an oxidation reaction and depends for its completion upon atmospheric oxygen.

As stated above, the alpha-H_2S positive type of colony results from the formation of sulfhemoglobin and the subsequent oxidation of the sulfhemoglobin to a green compound. Any change of environmental conditions affecting the production of hydrogen sulphide would also affect the production of the green coloration. In a medium consisting of 0.2 per cent ammonium sulphate, 0.1 per cent disodium phosphate, 0.5 per cent sodium chloride, 0.5 per cent glucose, and 2 per cent agar the alpha bacilli examined produced no hydrogen sulphide as determined by lead acetate. On blood agar plates made with this medium without the glucose the same alpha bacilli produced no green coloration and the colonies had the gamma appearance (see fig. 2, plate 1). In this same blood medium a number of strains of alpha streptococci and pneumococci also failed to produce a green coloration, although some hemolysis was produced (see fig. 4, plate 1). On chocolate agar made with this medium a beta-H_2O_2 positive streptococcus and some strains of alpha streptococci produced no green coloration, indicating that they were unable to produce any hydrogen peroxide on the medium. Addition of peptone to the synthetic medium generally restored the ability of the alpha bacilli to produce hydrogen sulphide, and of the alpha cocci to produce hydrogen peroxide. Different bacteria differ greatly in their ability to produce hydrogen sulphide or hydrogen peroxide on different media. The same can be said for the production of a green coloration. *Salmonella schottmulleri*, for example, will produce a good green coloration in extract blood agar, while *Eberthella typhosa* ordinarily will not do so. If infusion agar is used *E. typhosa* produces a green coloration. Many strains of *E. typhosa* which fail to produce a blackening of extract lead acetate agar may be found to blacken infusion lead acetate agar. If cysteine or sodium thiosulphate is added to infusion blood agar the alpha type of colony is formed by many bacilli which have the gamma type of colony on the standard infusion blood agar. In some cases even a change of peptone concentration from 1 to 2 per cent may be sufficient to change the appearance of an organism from that of a non-green-producer to a green-producer, the higher peptone concentration producing more green. The nature of the blood used, the concentration of salts, and the hydrogen ion concentration may materially effect the type of colony produced. Tunnicliff (1930) showed that some hemolytic streptococci will produce a green coloration on chocolate agar incubated at 32°C. but not at 37°C. The kind of agar used, and the manner in which the chocolate agar is produced also greatly influence this reaction. If the action of beta bacteria on chocolate agar is to be of any differential value, a standard technique for the preparation of chocolate agar must be adopted.

SUMMARY

A study of the effect of various bacteria on blood agar and on chocolate agar has resulted in the differentiation of five distinct types. The cause of the green coloration of blood agar has been determined with a fair degree of probability. The effect of various media and environmental conditions on the formation of the different types of colonies is discussed.

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PLATE I

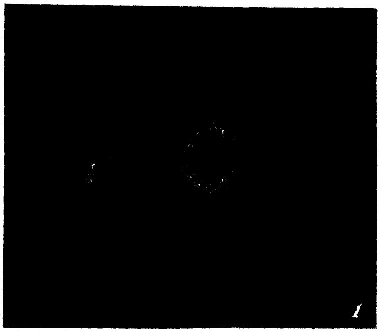
FIG. 1. Deep colonies of an alpha-colon bacillus in pork infusion blood agar after incubation at 37°C. for forty-eight hours. The green coloration is not apparent in the photograph but it is very strong. The hemolysis seems more prominent in the photograph than it appears to the naked eye.

FIG. 2. Same organisms as in figure 1 in synthetic blood agar. The colonies show no green coloration and practically no hemolysis

FIG. 3. Deep colony of a pneumococcus (alpha type) in pork infusion blood agar after incubation at 37°C. for forty-eight hours. To the naked eye the colony appears very hemolytic with a touch of green coloration.

FIG. 4 Same organism as in figure 3 in synthetic blood agar. The colony shows no green coloration but decided hemolysis.

FIG. 5. Same organism as in figure 1 in pork infusion blood agar to which was added 1 per cent sodium thiosulphate. The green coloration is much increased due to the addition of the thiosulphate.



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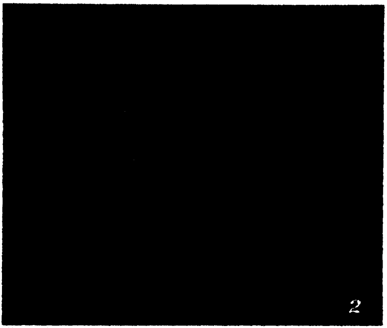

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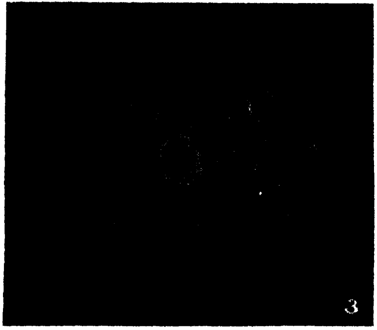

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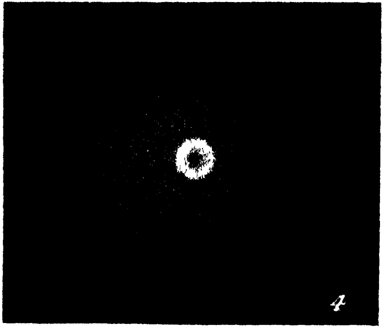

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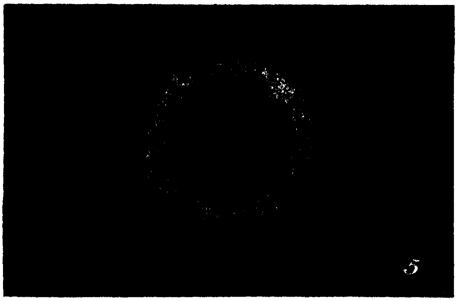

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(Einar Leifson Bacteria on Blood and Chocolate Agar)

PLATE 2

Fig. 1. Surface culture on chocolate agar of the same organism as in figure 1, plate 1. There is no green coloration.

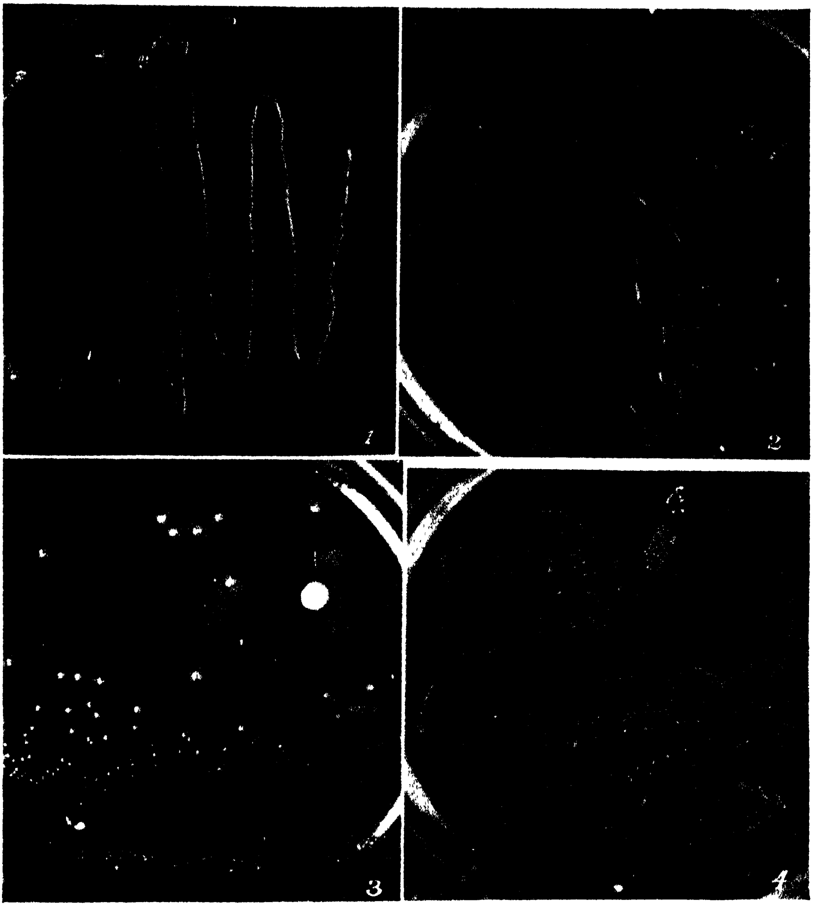
Fig. 2. Surface culture on chocolate agar of a pneumococcus (*alpha* type) showing a strong green coloration around the colonies.

Fig. 3. Surface culture on chocolate agar of a strain of *Str. epidemicus* (*beta*-H_2O_2 positive type) showing a green coloration around the colonies.

Fig. 4. Surface culture on chocolate agar of a streptococcus of the *beta*-H_2O_2 negative type showing no green coloration around the colonies.

The chocolate agar plates were all incubated at 37° C. for forty-eight hours.

For the photographs in plates 1 and 2 the writer is indepted to Dr. J. Howard Brown.



(Finn Tutson: Bacteria on Blood and Chocolate Agar)

THE PRODUCTION OF HYDROXYLAMINE BY THE REDUCTION OF NITRATES AND NITRITES BY VARIOUS PURE CULTURES OF BACTERIA[1]

GLEN A. LINDSEY AND CHESTER M. RHINES

North Dakota Agricultural Experiment Station

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Hydroxylamine can be prepared chemically by the oxidation of ammonia or by the reduction of nitrates or nitrites. This intermediary position upon the oxidation-reduction scale has led several workers to postulate that hydroxylamine is also an intermediary product in the oxidation of ammonia or in the reduction of nitrates by biological means. Proof of the presence of hydroxylamine in biological oxidations or reductions has, however, been lacking until the recent work of Blom (1928). He was able to demonstrate the production of hydroxylamine in several synthetic media in which the nitrate radical was the only source of nitrogen. The bacteria involved were an unknown mixture obtained from a large inoculum of soil. The present study was made in order to determine, by Blom's technic, if the ability to reduce nitrates to hydroxylamine is characteristic of a variety of organisms in pure culture.

Blom's test for hydroxylamine consists essentially of the determination of the nitrites formed by the oxidation of hydroxylamine with iodine. It is apparent that the medium must be free of nitrites before the presence of minute amounts of hydroxylamine can be demonstrated. None of the organisms used in the present study were able to remove all of the nitrites produced from nitrates when synthetic media similar to those of Blom's were used. In nutrient broth cultures, however, all of the nitrites formed were removed by the bacteria whenever the amounts of nitrates were sufficiently small. Some of the cultures reduced the nitrates and the nitrites with surprising rapidity, especially in the presence of d-glucose, as is shown by the following experiment. Sodium nitrate was added to twelve-hour d-glucose-broth cultures of *A. aerogenes* and *Esch. coli* at the rate of 27.5 mgm. per liter. At intervals of ten minutes the amount of sodium nitrite was determined colorimetrically by means of the sulfanilic acid-dimethyl-alpha-naphthylamine test. When the nitrites disappeared, the absence of nitrates was demonstrated by the diphenylamine and by the phenol-disulphonic acid methods. The results are shown in table 1.

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[1] Published with the approval of the Director of the Experiment Station.

TABLE 1

TIME	$NaNO_2$	
	A. aerogenes	*Esch. coli*
minutes	*mgm per liter*	*mgm per liter*
0	0	0
10	1 7	1 6
20	2 7	5.1
30	3 8	9.5
40	6 5	11 9
50	8 0	6 4
60	3 1	2 6
70	0	0

The test used for detecting small amounts of hydroxylamine was the same as the one used by Blom, except that dimethyl-alpha-naphthylamine was substituted for alpha-naphthylamine in accordance with the suggestion of Wallace and Neave (1927). The necessary reagents are as follows:

(1) 10 grams sulfanilic acid dissolved in 1000 cc. of 5 N acetic acid
(2) 6 cc. dimethyl-alpha-naphthylamine dissolved in 1000 cc. of 5 N acetic acid
(3) 1.3 grams iodine dissolved in 100 cc. glacial acetic acid
(4) 2.5 grams $Na_2S_2O_3 \cdot 5H_2O$ dissolved in 100 cc. of water

The procedure for the determination is as follows: Two cubic centimeters of solution 1 and a few drops of solution 3 are added to 10 cc. of the culture. After a period of five minutes, the excess iodine is removed with solution 4 and then 1 cc. of solution 2 is added. The development of a pink color is considered a positive test for hydroxylamine. About twenty minutes is usually required for the development of the maximum coloration.

The following organisms were studied: *A. aerogenes, Esch. coli, Staph. aureus, B. subtilis, B. ramosus, B. vulgatus, B. anthracoides, B. cereus, B. mycoides,* and *B. megatherium.* In all cases a sterile solution of sodium nitrate was added to actively growing broth cultures. At frequent intervals a nitrite test was made. When the last trace of nitrites disappeared, a hydroxylamine test was made. Frequently, in the cases where hydroxylamine was detected, a positive test could be obtained only during a period of a very few minutes after the nitrites disappeared. The time required for the reduction of the nitrates and nitrites varied with the amounts present and with the activity of the culture. Repeated trials with *Esch. coli* and *A. aerogenes* consistently gave positive tests for hydroxylamine when the tests were made immediately after the disappearance of the nitrites. The results were the same whether nitrates or nitrites were used. All of the other organisms which reduced nitrates and nitrites did so much more slowly than did *Esch. coli* or *A. aerogenes.* Positive results were obtained with cultures of *Staph. aureus, B. subtilis, B. vulgatus, B. ramosus,* and *B. anthracoides,* but not with perfect regularity. *B. cereus* and *B. mycoides* showed extreme weakness in their ability to reduce either nitrates or nitrites and no positive test for hydroxylamine was obtained. *B. megatherium* alone showed no ability to reduce nitrates or nitrites and always gave a negative test for hydroxylamine.

When hydroxylamine was added to the cultures in the form of the hydrochloride, it always disappeared very rapidly. In view of this fact, it seems probable that the cultures which reduce nitrites very slowly produce hydroxylamine at such a slow rate that it never accumulates in sufficient quantities to be demonstrated by the test used.

It is usually assumed that whenever the nitrite radical is reduced by bacteria, ammonia is formed. In a synthetic medium with sodium nitrate as the only source of nitrogen a luxuriant growth of *A. aerogenes* was obtained in twelve hours, yet the culture was ammonia-free, as judged by the Nessler test, until the fourth day. Since sodium nitrate was the only source of nitrogen, it naturally follows that the ammonia was produced by the reduction of the nitrates. Nevertheless, the intermediary stages in the process are not established. It is possible that during the first three days ammonia was formed by the reduction of nitrates through the intermediary steps of nitrites and hydroxylamine, but that it was utilized by the bacteria as rapidly as it was formed. On the other hand, it is also possible that no ammonia was formed during the first three days; and that the ammonia which appeared in the old cultures was a result of the degradation of the bacterial protoplasm.

CONCLUSIONS

Hydroxylamine has been demonstrated as a reduction product of nitrates and nitrites in broth cultures of *A. aerogenes*, *Esch. coli*, *Staph. aureus*, *B. subtilis*, *B. vulgatus*, *B. ramosus*, and *B. anthracoides*. The fact that these organisms represent such a diversity of types strongly suggests that the production of hydroxylamine is characteristic of the bacterial reduction of nitrites.

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